DETERMINING THE CALPAIN SYSTEM PROFILES OF BEEF, SPRINGBOK AND WILD GEESE USING THE SAME METHODOLOGY.

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Abstract – This paper describes a standardized method to quantitatively determine calpains and calpastatin activities in the muscles of various mammal and bird species. Firstly using three-step gradient ion-exchange chromatography; 0.0 M NaCl to partially separate calpastatin, 0.175 M NaCl to collect remainder of calpastatin and calpain-I, 0.35 M NaCl to collect calpain-II and then 1.0 M to regenerate column resin, then heating an aliquot of the co-elute calpain-I and calpastatin, and finally determining the activity and inactivity of the individual Ca^{2+}-dependent proteases quantitatively. Slight adaptions were made to this method to accommodate the determination of the calpain system profiles of beef, springbok venison and wild geese breast meat. The results showed that springbok longissimus had a similar calpain system profile than beef longissimus, but that the wild geese breast lacked calpain-I and from literature the calpain observed where calpain-II normally elutes is the µ-m-calpain that exists in bird species.

Key Words – beef, venison, wild geese breast (variety of species); calpain-system analyses method.

I. INTRODUCTION

There are a number of factors that play a key role in the ultimate tenderization process that occurs during *post mortem* storage of meat, one of which is the calpain proteolytic system [1]. Studies have been done to establish reliable methods and techniques to quantify and determine the activity of these components namely: calpastatin, calpain-I and calpain-II, and mostly in beef muscle [2, 3, 4, 5]. These methods have been adapted and perfected for the analyses of the calpain system in beef skeletal muscle, for many different research projects, at the Agricultural Research Council-Animal Production Institute (ARC-API), South Africa. There has been an increasing need to know the values of the calpain enzyme activities and calpastatin in the meat of various other species, to help explain the tenderization process or lack thereof. This methodology was applied with a few minor adaptations to apply the method to the analysis of springbok and wild geese.

II. MATERIALS AND METHODS

Experimental material

The calpain system profiles used for this paper was obtained from:

Beef samples were taken from *M. longissimus lumborum* of a typical South African beef breed, Bonsmara, which were reared in the feedlot of the ARC-API. Samples were collected 1 hour and 24 hours post-mortem [6]. Extra sample was collected 24 hours post-mortem to prepare calpain-II stock for calpastatin and calpain-I assays.

Springbok samples were taken from *M. longissimus lumborum* of the springbok project [7]. Mature springbok was harvested according to standard operating procedure at Elandsburg nature reserve Wellington, Western Cape, South Africa. Samples were collected 2 hours, 18 and 30 hours post-mortem.

Wild Geese samples were taken from breasts of the Egyptian geese [8] harvested on the University of Stellenbosch's experimental farm Mariendahl.

Sampling and storage of muscle sample

Samples of about 10 g were taken at the times indicated above and snap frozen in liquid nitrogen, and then stored at -80 ℃ until sample analyses. Samples were kept frozen until adding of extraction buffer.

Preparation of calpain-II stock for calpastatin and calpain-I assays (on ice):

Extract was prepared from extra sample collected (6 x 4 g) in 15 ml extraction buffer described below for analized samples. Procedure is the same as for normal samples except that all 6 samples were combined into one glass beaker without dilution. Combined sample was loaded on a 20 ml DEAE Sepharose (GE Heathcare Bio Sciences AB, Upsala, Sweden) packed column. Calpastatin was removed from sample with 50 ml 0.04 M Tris HCL pH 7.5; 5 mM EDTA, 0.025% MCE elution buffer

containing 0.15 M NaCl. Calpain-II is collected via a fraction collector after elution with 100 ml buffer containing 0.35 M NaCl. The column resin was washed with 50 ml 1.0 M NaCl and regenerated with 100 ml elution buffer containing no NaCl. Every test tube containing sample fractions collected were tested for calpain-II activity with the azo-casein assay and all fractions that had an absorbance $\geq$ 0.100 than the blank at 366 nm were collected and used to determine the activity of calpastatin and indirectly the calpain-I.

Separation and calculation of calpastatin, calpain-I and calpain-II in beef muscle (on ice):

Extraction of samples was done according to an adapted method of [4]. Frozen muscle (3 g), was homogenised in 15 ml extraction buffer (75 mM Tris HCl, 10 mM EDTA, pH 7.8, 0.05% [vol/vol] 2-mercaptoethanol (MCE), 2 mM phenylmethylsulfonyl fluoride (PMSF), 1 ul/L pepstatin A, at 4 °C) and centrifuged at 10 000 x g for 30 min, filtered through vitrace, pH adjusted to 7.5 and volume made up to 20 ml with extraction buffer.

The separation of calpastatin, calpain-I and calpain-II was based on the three-step gradient ion exchange chromatography-method of Geesink & Koohmaraie [3]. The Gilson LPLG system (pump, DEAE Sepharose column, valve mate, fraction collector; Gilson Medical Electronics, Inc. 3000W Beltline Hwy, Middleton, USA) with the automatized software program (Unipoint™ LC version 5.1) were applied to separate the calpastatin and calpain-I from the calpain-II with NaCl-Tris/HCl buffers at pH of 7.5. After loading the 20 ml extract the column was eluted with 0.0 M NaCl (to partially separate calpastatin), 0.175 M NaCl (to collect remainder of calpastatin and calpain-I, 0.35 M NaCl (to collect calpain-II) and then 1.0 M (to regenerate column resin). Calpastatin activity was determined in each of the 0 M and 0.175 M NaCl pooled elutes. Calpain-I activity was determined indirectly by estimated calpastatin activity before and after heat treatment of the 0.175 M salt fraction. A 5 ml aliquot from the pooled 0.175 M NaCl eluent containing both calpastatin plus calpain-I was heated for 15 min at 95 °C, cooled on ice and centrifuged at 4000 x g, for 15 min to remove precipitate to eliminate calpain activity. Calpain-II activity was determined in the pooled fractions of 0.35 M NaCl elute directly as described in Azo-casein assay.

The activity of calpains were determined using the *Azo-casein assay* as described by Dransfield [4]. The use of azo-casein eliminates the problem of background absorbance of non-specific proteins in the extracts. In short the assay buffer contains 7.5 ug/ml azo-casein in 0.1 M Tris/HCl buffer, pH 7.5 containing 0.1 M $CaCl_2$, and 0.05% (vol/vol) MCE at 4 °C. Reaction is started by adding 400 µl "sample" (prepared as specified below) to 400 µl assay buffer and incubate at 25 °C. The reaction was stopped after 1 hour with 400 µl of 10% trichloroacetic acid (TCA) and the reaction mixture centrifuged at 4000 x g. The absorbance of the supernatant was determined at 366nm (Spectrophotometer: Beckman Coulter (DU 730 life science UV/Vis spec).

A triplicate of each of the following were prepared:

1. Blank: replacing sample with 400 µl extraction buffer;
2. Stock calpain-II: 100ul + extraction buffer: 300 µl;
3. 0.0 M NaCl fraction (calpastatin only): 100 µl + Stock calpain-II: 100 µl + extraction buffer: 200 µl;
4. 0.175 M NaCl fraction (calpastatin plus calpain-I): 100ul + Stock calpain-II: 100 µl + extraction buffer: 200 µl {before heat};
5. 0.175 M NaCl fraction (calpastatin plus calpain-I): 100ul + Stock calpain-II: 100 µl + extraction buffer: 200 µl {after heat};
6. 0.35 M NaCl fraction (calpain-II): 400ul.

The calpain-I activity was estimated by subtracting calpastatin activity in the 0.175 M NaCl fraction before and after heat treatment. The calpain-I activity can be determined within a 10% margin of error [3]. One unit of calpain activity is defined as an increase in absorbance of 1.0 at 366 nm per hour at 25°C.

Calpastatin activity was determined adding the same quantity of extracted calpain-II stock to each assay (component) against a blank prepared from assay buffer which was performed in triplicate. One unit of calpastatin activity was defined as the amount that inhibited one unit of m-calpain activity divided by two. Calpastatin values were divided by 2 because of the nature of the activity reaction of the calpastatin molecule with combination of the calpains (I and II) molecules [5]. Data is expressed as units per gram of muscle or units per milligram of extractable protein (specific activity). The calpain and calpastatin determinations as described above are considered to be estimates and not exact determinations, as

they are influenced by numerous factors such as protein extractability, the inseparability of calpastatin and calpain I and enzyme stability.

Calpastatin, calpain-I and calpain-II determinations in Springbok muscle – some adaptions:

Extra sample were not collected to prepare calpain-II stock for determining calpastatin activity. The beef method was followed exactly as described above except that the calpain-II collected in the 0.35 M NaCl elute was used to determine the calpastatin activities as described above and 200 µl calpain-II instead of 100 µl as the activity of calpain-II elated from the columns was not as high than that prepared separately and an adequate level of activity is necessary to determine calpastatin levels in the assays. The same calculations formulas were used as for the beef method.

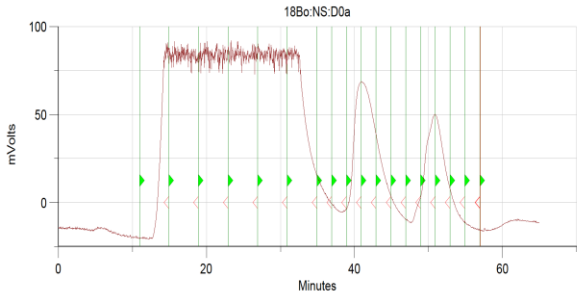

Figure 1. Typical profile achieved from the Unipoint software programme after elution with 0.0 NaCl, 0.175 M NaCl and 0.35 M NaCl buffers.

Calpastatin and µ-m-calpain determinations in Wild geese muscle – some adaptions:

The beef method was applied as described above. The 0.0 M NaCl elution peak showed minimal calpastatin activity and the 0.157 M NaCl elution peak showed calpastatin activity and no calpain-I activity using above described approach. Calpain activity was found in the 0.35 M NaCl. This indicated that no calpain I activity was present in the sample. The proteolytic activity detected in the 0.35 M NaCl Tris HCl buffer, pH 7.5 with the azo-casein assay is therefore attributed to µ/m-calpain according to [9].

Protein concentration of the LL frozen samples was determined by the Buiret method of Cornall, Bardawill, and David [10].

Table 1. Typical calpain system activity results for different species; beef [6], springbok [7] and wild geese [8] determined at different times post-mortem.

Beef	Hours post-mortem	
Longissimus	1	24
Calpastatin	2.329^{a}	2.022^{b}
	(0.047^{a})	(0.040^{b})
Calpain-I	1.757^{a}	1.497^{b}
	(0.035^{a})	($0.030^{b)}$
Calpain-II	1.129	1.152
	(0.023)	(0.023)
Calpastatin/Calpain-I	1.373^{a}	1.427^{b}
Calpastatin/Calpain-I + Calpain-II	0.819^{a}	0.764^{b}

Springbok	Hours post-mortem		
Longissimus	2	18	30
Calpastatin	1.434^{a}	1.365^{ab}	1.198^{b}
	(0.023^{a})	(0.022^{ab})	(0.019^{b})
Calpain-I	1.116^{a}	0.933^{b}	0.754^{c}
	(0.018^{a})	(0.015^{b})	(0.012^{c})
Calpain-II	0.966	0.977	0.935
	(0.015)	(0.015)	(0.015)
Calpastatin/Calpain-I	1.345^{a}	1.727^{b}	2.263^{c}
Calpastatin/Calpain-I + Calpain-II	0.689^{a}	0.726^{b}	0.737^{b}

Wild Geese	Hours post-mortem			
Breast	0.15	1.15	2.15	3.15
Calpastatin	1.094^{a}	0.791^{b}	0.627^{c}	0.548^{cd}
	(0.018^{a})	(0.013^{b})	(0.010^{bc})	(0.009^{cd})
µ-m Calpain	2.077^{a}	2.137^{b}	2.246^{c}	2.199^{bc}
	(0.034)	(0.035)	(0.036)	(0.034)
Calpastatin/µ-m Calpain	0.536^{a}	0.366^{b}	0.273^{bc}	0.245^{bc}

[1]One unit of calpastatin activity was defined as the amount that inhibited one unit of m-calpain activity divided by two.
[2]One unit of calpain activity is defined as an increase in absorbance of 1.0 at 366nm per hour at 25°C.
[3] Values in brackets represent specific activities.
[a,b,c,d] Means within a row with different superscripts differ significantly ($P<0.05$).

III. RESULTS AND DISCUSSION

Table 1 represents results typically for the species analyzed by means of the procedure described above. A few manuscripts presenting beef calpain system data were published with data acquired from the procedure under discussion for example

Strydom and Frylinck [11], Strydom et al. [12], Frylinck et al. [13] and Hope-Jones et al. [6]. More recently the demand was to analyze calpain system profiles of other species such as springbok and wild geese. Especially the wild geese analyses presented challenges when the normal data patterns were not achieved. The 0.0 M NaCl elution peak showed minimal calpastatin activity indicating towards differences in molecular characteristics. The 0.175 M NaCl elution peak showed calpastatin activity and but no calpain-I activity. This is in agreement with the literature, where it has been reported that calpain I as described for mammalian species is not present in avian muscle, instead a different calcium-dependent proteinase known as μ/m-calpain is present 9]. Calpain activity was found in the 0.35 M NaCl. Recently an article was accepted for publication presenting wild geese calpain system data [8]. Calpain system profiles achieved for springbok was similar to that of beef and the normal calpastatin, calpain-I and calpain-II could be identified. Harvesting challenges resulted in samples only taken at earliest 2 hours post-mortem and a direct comparison with beef is not possible. However the levels of the different calpain system components seem to be lower than that of beef.

IV. CONCLUSION

From the results obtained this method can be applied to a number of different species with a few minor adaptations with reliable results.

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