ON MAKING IT FEASIBLE TO ANALYZE AND RELATE THE ACTIVITIES OF LYSOSOMAL PROTEASES NON-DESTRUCTIVELY TO THE FINAL QUALITY OF DRY CURED HAMS

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Introduction

Cutting of the ham before salting is critical as it determines important characteristics like direction and time needed for salt and water diffusion. Trimming of raw hams before processing is therefore a standardized procedure. The trimming generally makes it possible to collect small pieces of muscles, some from highly relevant ham muscles, for future characterization of the raw material.

In an ongoing project (Thauland et al., 2003), where the method of X-ray computed tomography is developed for interval monitoring of salt content and distribution in ham, the small pieces from *Gluteus medius* (GM) and *Semimembranosus* (SM) were collected as possible future indicators of the final quality of dry cured hams. As the enzyme activity is salt dependent, relevant assay should be carried out once the correct salt concentration is known for a specific dry cured ham. The suitability of the trimmings for lysosomal enzyme activity determination was evaluated here as the activities of such enzymes have been indicated as important determinants of end quality of dry cured hams produced over longer (9-12 months) time periods (see for example Garcia-Garrido et al., 2000).

At the same time some aspects related to using a fluorescence assay for enzyme analysis in a quantitative manner on widely different (age and breed) animals were studied.

The aspects studied were: the effect of myoglobin absorbance and the light scattering from the fat present in the collected muscle pieces on the emitted fluorescence intensity.

Objectives

The objectives were to examine the suitability of the small muscle pieces (GM and SM) trimmed from widely different green hams, for lysosomal enzyme assays.

Methodology

Materials: 55 large hams (11-14 kg) were bought from a slaughterhouse as regular hams with shank, and trimmed for dry cured ham production by expert trimmers at The Norwegian Meat Research Center. Pieces of SM and the lighter part of GM were collected and weighted.

In addition, pieces from 16 widely different hams, removed as above, were used for determination of enzyme activity in a screening experiment. These samples were selected from different breeds (Norwegian Landrace/Yorkshire; Noroc (=Norwegian Landrace/Yorkshire/Duroc) and Duroc) and ages (5-36 months). Obvious fat tissue was removed from meat samples. 5 g meat samples, frozen in liquid nitrogen, stored at -80°C were analyzed during a 6 week period.

Methods:Enzyme assay: The enzyme (cathepsin B and B+L) activities were analysed according to the principles of Etherington et al. (1987) and Parreño et al. (1994). Extraction buffer (0.05 M sodium citrate buffer with 1 mM Na_2-EDTA and 0.2% w/v Triton X-100, pH 6.0) to meat was 10:1. Homogenisation: 3 x 30 sec using polytron. Standard centrifugation lasted 15 min at 4°C at 21 000 g_{max}. Thereafter filtration was performed using Whatman filter 4. This extract was used for scatter determination; for several measurements of total protein content, and for some myoglobin and cathepsin determinations. Extended centrifugation lasted 1 hr at 21 000 g_{max}, and then only the extract below the upper lipid layer was used. Essentially this extended centrifugation method removed most of the lipid material from the extract. Cathepsin B and B+L were assayed with substrates N-CBZ-L-arginyl-L-arginine-7-amido-4-methylcoumarin (Z-Arg-Arg-Nmec, Sigma Aldrich C5429)) and N-CBZ-L-phenylalanyl-arginine-7-amido-4-methylcoumarin (Z-Phe-Arg-Nmec, Sigma Aldrich C3282)), respectively. NMec was used as standard (Sigma Aldrich, A9891). Substrates and standards were used at 5 μM; the assay was at 37°C for 30 min (cathepsin B) or 25 min (cathepsin B+L), respectively. *Total protein:* Protein was determined by the biuret method (http://www.hamline.edu/depts/biology/courses/biocon2/biuret.html) using bovine serum albumin (96 % pure) as standard. Total protein concentrations were used to calculate specific enzyme activities.

Myoglobin concentration: Myoglobin concentration was estimated using the absorbance of the extracts at 525 nm after extended centrifugation. Commercial myoglobin (Sigma Aldrich, MO630-16) was used as standard. *Light scattering:* The absorbance (525 nm) before and after extensive centrifugation, was taken as scattered light. *Instrumentation*: Perkin Elmer LS 50 was used; excitation was at 342 nm, bandwidth 2.5 nm; scanning rate 250 nm/min. Large cuvettes were used. Spectral measurement was at 21.5 °C. Spectra from 360-600 nm were acquired.

Statistics: The computer program from MINITAB Statistical Software (version 14.12; Minitab Inc., State College, USA) was used to perform one-way analysis of variance, one-way multiple comparisons of mean, paired tests and regressions

Results & Discussion

Figure 1 shows a picture of a ham trimmed for production of dry cured ham. The muscles SM, BF (*Biceps femoris*) and the lighter part of GM are indicated in Figure 1. **Table 1** gives the weights of the three different muscle pieces. It is apparent that the

amount of SM removed can be very small. The variation in size was, however, large. Priority was given to comparing the enzyme activities of GM and SM as SM is the larger muscle inside the dry cured ham, while GM was easily assessable in large quantities. In addition, the activities of BF and SM have been compared earlier (Parreño et al., 1994).

Table 2 gives scattered light, myoglobin content and enzyme activities for the 16 extracts analyzed. Light scattering seemed due to lipid-emulsification during work up of the extract and could be difficult to remove. Light scattering was typically most problematic with the Noroc/Duroc breeds; breeds known for their higher levels of intramuscular fat. Myoglobin level was significantly related to animal age ($p<0.05$). Total protein concentrations of the extracts differed; SM having the higher mean of total protein (SM: 7.7 mg/ml; GM: 7.1 mg/ml; $p< 0.01$).

Two SM muscles were selected for setting up the enzymatic assay (**Figure 2**). These muscles were chosen because they gave the higher amount of myoglobin in the extract (animal 1; Table 2) and the higher amount of light scattering during standard work-up procedure (animal 2; Table 2). These muscles were characterized by being 1) from an older animal and 2) of the breed Duroc with a high intramuscular fat content, respectively. Myoglobin absorbance was severe only for wavelengths below 460 nm when the meat extraction fraction (meat extract: total assay solution) was $\leq$ 16.2% (Figure 3). As an example, the reduction in emission intensity at 460 nm using a myoglobin concentration of 0.026 mg/ml was 6%. Scattering (Figure 2, animal 2), if present, seemed prominent at all wavelengths. It could be surmised that the actual position from where the SM sample was obtained, would make it difficult to remove all fat (see Figure 1).

The presence of absorbance and scattering will also reduce the possibility of obtaining a high correlation between the disappearance (at 360 nm) of emission from the substrate and the appearance (at 460 nm) of emission from the product. In our case the correlation was $r= 0.82$ and $r=0.69$ for GM and SM, respectively, when meat fractions up to 16.2% were used. Lack of a better relationship is suggested to be due to analytical errors accompanying the assay when working at low muscle extract fractions, and bias due to scattering and absorbance. It should be memorized that the impact of myoglobin absorbance cannot be removed from the assay, unless myoglobin, or any other interfering pigment, is removed from the extract for example by chromatography.

The fate of the exciting light in terms of scattering/absorbance/emission will of course depend on the ratio between fluorochrome and muscle extract. However, this ratio can only be controlled at the beginning of the assay.

The enzyme analysis (Table 2) were therefore carried out with meat extract fractions $\leq$ 4.2%. This low fraction will secure that the effect of myoglobin absorbance should not affect the emission spectrum too much at 460 nm; a wavelength typically used for measuring enzyme activity.

No significant difference in the mean enzyme activities of cathepsin B and B+L for the 16 samples of the two muscles was found. The mean activities being 2.5 µM x min^{-1}x g^{-1} and 3.9 µM x min^{-1}x g^{-1} for cathepsin B and cathepsin B+L, respectively (mean specific activities were 0.37 and 0.54 µM x mg^{-1} protein, respectively).

In contrast, significant differences between animals were detected (Table 2). Two Noroc breeds had significantly higher cathepsin B in GM than the other animals had; no significant differences were found for SM from the different animals. For cathepsin B+L

it also seemed more difficult to obtain a significant differentiation between animals using SM compared to GM. However, significant differences between animals were found for both muscles (Table 2).

A larger experiment involving X-ray computed tomography analysis throughout long term processing of hams is now underway. The experiment that will be supported by the biochemical methods described above.

Conclusions

The *mean* cathepsin B and B+L activites determined on meat pieces removed from *Semimembranosus* and *Gluteus medius* of 16 different animals were not differentiated.

The small pieces of *Semimembranosus* removed from Noroc/Duroc breeds were difficult to measure due to light scattering, and it was also more difficult to differentiate between animals.

The highest cathepsin B activity was measured in *Gluteus medius* from a Noroc breed.

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Tables and Figures

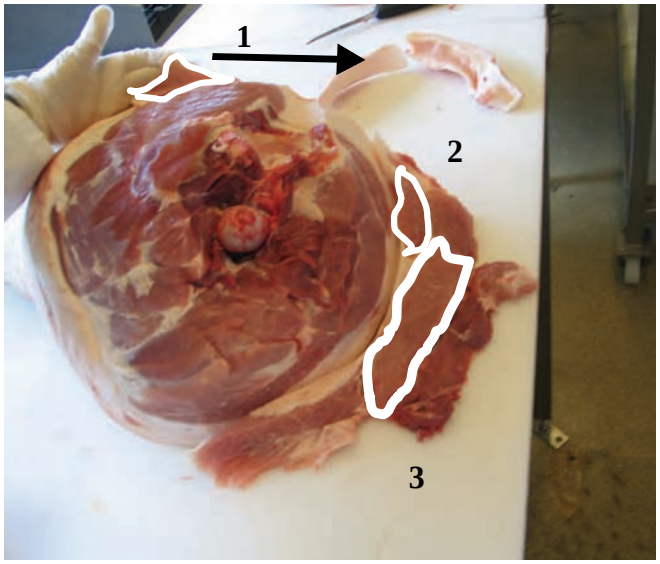

Figure 1. The figure shows a ham trimmed for dry cured ham production. Smaller parts of three muscles were collected. These muscles were 1) Semimembranosus 2) Biceps femoris and 3) Gluteus medius (lighter part). The arrow points at the origin of Semimembranosus, i.e. just beneath the surface of the subcutaneous fat.

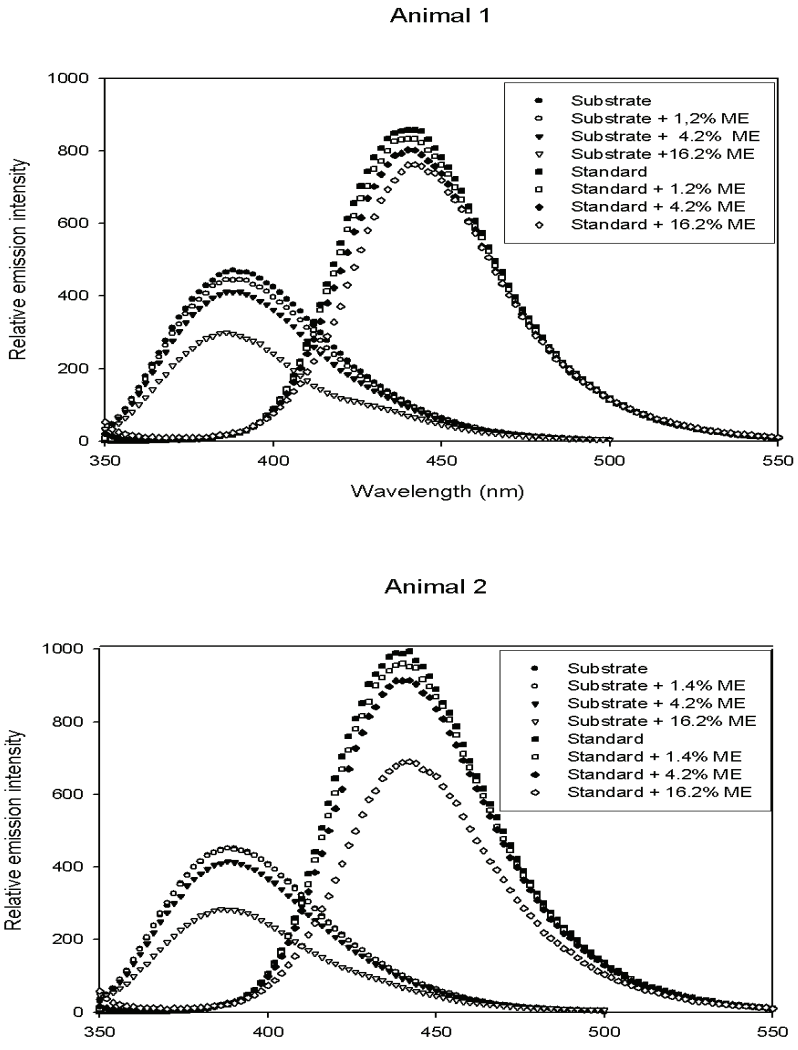

Figure 2. Emission spectra of substrate (to the left) and standard (to the right) obtained for two (1, upper panel; 2, lower panel)) different animals as a function of muscle extract (ME) fraction. The enzyme activity was stopped by the presence of 0.72 % mono chloroactetate.

Table 1. Weight of pieces of muscle removed upon trimming 55 hams for dry cured ham production.

Muscle	Mean (g)	Standard deviation (g)	Minimum (g)
Gluteus medius	208	97	107
Biceps femoris	75	32	21
Semimembranosus	39	20	7

Table 3. The characteristics of the 16 different meat pieces from *Semimembranosus* (SM) and *Gluteus medius* (GM) that were screened for lysosomal enzyme activity (*expressed as activity in µM x min^{-1}x g^{-1} meat sample).

The same letters, by row, indicate no significant difference (p> 0.05).

Breed	Landrace/ Yorkshire	Duroc	Landrace/ Yorkshire	Landrace/ Yorkshire	Landrace/ Yorkshire	Landrace/ Yorkshire	Landrace/ Yorkshire	Landrace/ Yorkshire	Noroc	Noroc	Noroc	Noroc	Noroc	Landrace/ Yorkshire	Landrace/ Yorkshire	Landrace/ Yorkshire
Age(yrs)	3	0.42	1.5	1.5	1.5	1.5	1.5	2	0.42	0.42	0.42	0.42	0.42	0.42	0.42	1.5
Animal number	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16
Scattered light-SM	0.008 [a]	0.469 [c]	0.077 [a]	0.001 [a]	0.062 [a]	0.02 [a]	0.003 [a]	0.03 [a]	0.391 [c]	0.089 [b]	0.308 [c]	0.271 [c]	0.029 [a]	0.043 [a]	0.038 [a]	0.067 [a]
Scattered light-GM	0.019 [a]	0.21 [c]	0.066 [a,b]	0.009 [a]	0.068 [a,b]	0.019 [a]	0.011 [a]	0.095 [b,c]	0.243 [c]	0.099 [a,b]	0.136 [b,c]	0.251 [c]	0.052 [a]	0.043 [a]	0.046 [a]	0.024 [a]
Myoglobin(mg/ml)-SM	0.45 [c,d]	0.29 [b]	0.41 [c,d]	0.29 [b]	0.43 [c,d]	0.37 [b,c]	0.29 [b]	0.48 [d]	0.36 [b,c]	0.32 [b,c]	0.35 [b,c]	0.26 [a,b]	0.35 [b,c]	0.29 [b]	0.29 [b]	0.37 [c,d]
Myoglobin(mg/ml)-GM	0.37 [b,c]	0.26 [a]	0.39 [c,d]	0.40 [c,d]	0.40 [c,d]	0.35 [b,c]	0.34 [c]	0.37 [b,c]	0.32 [b]	0.32 [b]	0.33 [b]	0.34 [b]	0.37 [b,c]	0.26 [a]	0.28 [a]	0.39 [c,d]
Cathepsin B activity-SM*	1.43 [a]	2.30 [a]	1.99 [a]	1.38 [a]	0.87 [a]	1.51 [a]	1.67 [a]	2.19 [a]	3.09 [a]	3.45 [a]	2.02 [a]	2.92 [a]	1.45 [a]	4.47 [a]	4.58 [a]	1.57 [a]
Cathepsin B activity-GM*	1.20 [a]	2.18 [a,b]	1.51 [a,b]	1.70 [a,b]	1.36 [a]	1.29 [a]	1.65 [a,b]	1.71 [a,b]	2.30 [a,b]	9.07 [b]	4.58 [b]	3.56 [a,b]	2.66 [a,b]	1.43 [a]	2.38 [a,b]	2.12 [a,b]
Cathepsin B+L activity-SM*	2.39 [a]	4.49 [a,b]	3.32 [a,b]	2.05 [a]	1.91 [a]	3.73 [a,b]	3.34 [a,b]	3.91 [a,b]	3.83 [a,b]	7.83 [b]	5.03 [a,b]	5.56 [a,b]	5.75 [a,b]	4.17 [a,b]	4.15 [a,b]	2.72 [a,b]
Cathepsin B+L activity-GM*	2.21 [a]	4.45 [a]	3.76 [a]	4.98 [a]	2.00 [a]	1.78 [a]	3.32 [a,b]	3.81 [a,b]	3.91 [a,b]	2.93 [a,b]	4.64 [a,b]	5.25 [a,b]	6.83 [b]	2.86 [a,b]	6.86 [b]	4.75 [a,b]