INFLUENCE OF THE TYPE OF DRY-CURED ITALIAN PDO HAM ON CATHEPSIN B ACTIVITY DURING PROCESSING

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Abstract – Cathepsin B activity was measured in hams originating from the main Italian *prosciutto* PDOs: Parma, San Daniele and Toscano. Sixty homogeneous heavy pig thighs were distributed in one plant per PDO, and sampled at *Biceps femoris* in groups of four per plant at: salting, resting, drying, greasing, end of curing. The ripening trend of cathepsin B activity (U/g protein; measured by fluorescence) differed significantly among the various PDOs, particularly during the initial and mid curing stage. This activity correlates with the proteolysis index through a PDO dependent pattern, indicating that the different processing conditions can influence the quality of prosciutto, since they determine its biochemical development.

Key Words – Parma, San Daniele and Toscano *prosciutto*, lysosomal proteases, activity trend

I. INTRODUCTION

The most important Protected Designations of Origin (PDOs) for Italian dry-cured ham (*Prosciutto*) are: Prosciutto di Parma (PA), Prosciutto di San Daniele (SD) and Prosciutto Toscano (TO), which produced 9.8, 2.7 and 0.27 million conforming matured prosciuttos, respectively, in 2009. The processing conditions of the three PDO hams differ. SD is the only one which retains the trotter. In the dressing phase, TO is cut in a "V" shape by removing a greater part of the skin than the other two PDOs, for increasing the penetration of salt into the ham. The salt can be added to the three PDOs in different forms: rubbed in dry and wet for PA, dry and ad libitum for SD, dry and mixed with pepper and vegetable flavourings for TO. The 'ripasso' i.e. the replacement of the old salt with new during salting, is only performed once for PA and SD and twice for TO. In the pre-resting phase, SD is pressed into the typical guitar shape, facilitating the penetration of the salt; at greasing, for TO, the classic 'sugna' (leaf fat mixed with wheat or rice flour) is supplemented by pepper and vegetable flavourings. In ham maturation process the cathepsins perform an essential role, particularly B, D and L which, together with H, represent the most abundant lysosomal proteases in the muscle. Cathepsin B (cB) appears to be particularly active in relation to desmin, myosin, actin and tropomyosin I [1] and it is particularly stable compared with other cathepsins throughout the ham processing [2]. Moreover, green thighs with high activity of cB have been reported to yield more proteolysed PA dry-cured hams [3]. Anyway, the transformation of fresh thighs into dry-cured ham interferes with its enzymatic activity; indeed, various authors have noted that the salt concentration, temperature, water activity and pH interfere with cB [2,3,4,5]. The purpose of this study is to investigate the influence of the production process on enzymatic activity in industrial conditions, measuring its variation in different processing phases in hams from three Italian PDOs. To our knowledge, this is the first time SD and TO cB trend in seasoning has been examined and compared with PA.

II. MATERIALS AND METHODS

The test material comprised 65 heavy pig thighs, in line with the PDO requirements for conformity, from 65 Large white x Landrace of Italian pigs, bred on a single farm with the same balanced diet, and slaughtered in the same abattoir. Five thighs represented the initial control, time 0 (T0). The other 60 were distributed to three prosciutto plants belonging to the three different PDOs, in accordance with a balanced distribution plan. Each PDO plant thus received 20 thighs, the weights of which were recorded before and after dressing. The ripening process was monitored, and the days since receipt recorded in the following phases: T0, slaughter; T1, out of salting (salting); T2, introduction to the resting room (resting); T3, after

washing and drying (drying); T4, mid-curing, after greasing (greasing); and T5, end of curing (curing). Four thighs were sampled per prosciutto plant in each T1 to T5 phases. The weight losses net of trimming subtractions and additions of sugna, i.e. an estimate of the water losses on the specific phase of ripening (in g/kg of dressed-thigh weight), were calculated. A sample of *Biceps femoris* muscle (BF) of around 100g was taken from the thigh for the determination of moisture and sodium chloride content; proteolysis index (PI): the percentage ratio of Non Protein Nitrogen (proteins precipitated by 20% (w/v) trichloroacetic acid) on Total Nitrogen; water activity (a_w; AquaLab Dew Point Water Activity Meter 4TE, Decagon Devices, Inc., Pullman, WA, USA); protein content by Lowry's assay; and cB activity.

The cB extraction was carried out suspending 300 mg BF in 3 ml extraction buffer comprising 50 mM Na citrate, 1 mM Na2-EDTA, 0.2% Triton x-100 with pH 5.0. The solution was homogenized twice for 20 sec with Ultra-Turrax® T25 Digital, and centrifuged for 20 min, 11000g at 4 °C. The supernatant was used to measure both cB activity and protein content. The cB activity was determined by fluorescence measurements, using Z-Arg.Arg-7-amido-4-merhylcoumarin hydrochloride (Z-RR-AMC; Sigma-Aldrich S.r.l. Milan, Italy) [1]. The measurement was performed using a microplate spectrofluorometer (Molecular Devices Inc., Sunnyvale, CA, USA) with emission and excitation of 355 nm and 460 nm respectively. In each sample containing 2.5 µM Z-RR-AMC and 50 mM sodium phosphate buffer pH 6.0, 4 mM EDTA, 2 mM DTT and 3.4 ml/L Brij 35 in 200 µl [2], 0.2-1.6 mU of cB (Sigma-Aldrich S.r.l., Milan, Italy) or 20 µl of extract were added. The kinetics was monitored for 30 min, at 37 °C. The cB activity was calculated as micromoles of AMC released per min at 37 °C, thus expressing the activity in U/g protein or in U/g wet muscle.

The statistical analysis was performed by the GLM procedure of the SPSS package, v. 17 (SPSS Inc., Chicago, IL, USA). A multiple covariance analysis was performed for 60 independent hams cured for a different period of days (D), where the individual hams represent three categories (PA, SD and TO) of the factor 'PDO ham type' and D, D^2, D^3, D^4 the covariates, that were added sequentially, by the forward selection method. The purpose of the multiple covariance analysis was to verify whether the multiple regression line of the ham characteristic on the respective day of processing was significantly different for PA, SD and TO. If the covariance coefficients differed significantly, a one-way ANOVA was performed for the relevant characteristic, independently for each processing phase, in order to compare the PDO ham types in the five examined phases.

III. RESULTS AND DISCUSSION

The weight of the thighs after dressing was higher for SD than TO (14.43 *vs.* 13.59kg, $p<0.05$), with PA having an intermediate value (14.20kg). These differences are in line with the dressing procedures, which involve retention of the trotter in SD prosciutto and more extensive Semimembranosus skinning in TO prosciutto.

Table 1. Whole ham water loss and BF salt content during five curing phases (see text) of PDO hams

Curing	Water loss (g/kg)				Salt content (%)			
phase	PA	SD	TO	PSD	PA	SD	TO	PSD
T1	25^b	26^b	47^a	6.5	1.0	1.0	1.0	0.01
T2	116	108	108	14.4	1.2	1.7	2.0	0.68
T3	157^b	198^a	188^a	12.1	2.3^b	2.8^b	4.1^a	0.57
T4	227^b	247^{ab}	272^a	21.8	3.9^b	4.0^b	5.2^a	0.66
T5	284^b	270^b	307^a	17.0	5.6^b	6.0^b	9.6^a	0.38

^{a,b,c}: PDO differences within a curing phase $p \leq 0.05$;
PSD: pooled standard deviation.

As expected [5], the processing conditions of the three Italian PDOs had a significant influence on the final physical-chemical characteristics of the prosciutto (Table 1). TO emerges as the one that underwent the greatest losses of water and a_w (from 0.96 in T1 to 0.86 in T5, data not tabulated), and had the lowest moisture level (54.8% at curing, data not tabulated) and the highest content of NaCl. These data are consistent with a production process which, entailing more intense trimming and increased salting, favours a greater water loss and more intense assimilation of salt. At the end of curing, PA and SD prosciutto were characterized by comparable values of moisture (60.5% vs. 61.1%), a_w (0.91) and salt content.

Figure 1 shows the PDO dependent change of PI during the various phases of processing. It is interesting to note that from greasing PA presented a significantly higher proteolysis than the other two PDOs. At the end of ripening, PI reached the

values of 28.6%, 24.6% and 22.4% (p<0.05) in PA, SD and TO respectively.

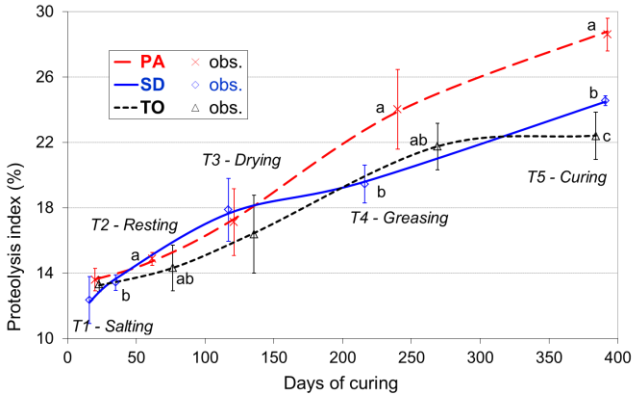

Figure 1. Trend of PI in BF during curing of PDO hams (covariate model curves and observed mean, obs., with standard deviation values; a,b,c: differences between PDOs within a ripening phase $p \leq 0.05$)

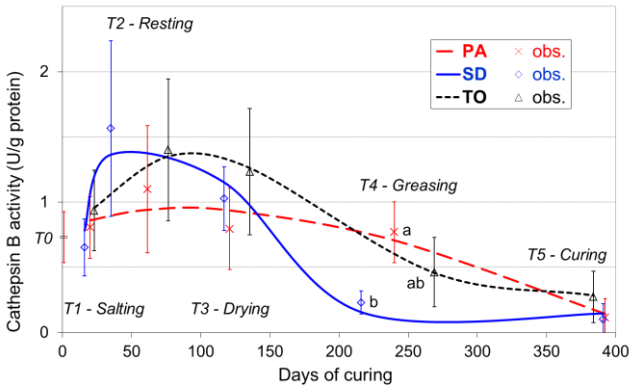

Figure 2. Trend of cB activity in BF during curing of PDO hams (covariate model curves and observed mean, obs., with standard deviation values; a,b,c: differences between PDOs within a ripening phase $p \leq 0.05$)

Figure 2 shows the temporal trend of cB activity. At T0, the average activity in the fresh thighs was 0.73U/g protein, which corresponds to 0.057U/g wet weight. This value falls towards the lowest end within the range of activity recorded in the literature, which is, however, very broad [1,4,6]. From this quite low value, one could therefore assume that in the fresh meat, where the lysosomal structures are now damaged, a high percentage of enzyme is bound to its inhibitor cystatin [4]. In the drying phase (T3), a decrease in activity could be noted in comparison with T2 in all PDOs, chiefly in SD and TO. A comparable trend, ascending in the first phases and descending in the successive phases, had already been shown, although not correlated with the processing phases [6,7], and it may be attributable to the progressive dehydration and decrease in the a_w of the muscle. This marked decreasing trend continued for SD and TO ham during the first curing period, until sugnatura. In T4, cB activity in the three PDOs differed significantly. SD presented the lowest activity (0.23U/g protein), while PA showed the highest activity (0.77U/g protein), and TO had an intermediate activity between PA and SD (0.46U/g protein). Finally, in T5 at the end of ripening, all of the cured hams showed a distinctly lower activity in comparison with that originally measured in the fresh meat (T0), and were not dissimilar to one another. At this time, however, the proteolysis values differed among PDOs. PA had the highest values, probably as these are cumulative and a consequence of the high level of cB activity in T4.

To better understand the role of cathepsin B, its cumulative activity (cBc) was calculated as the definite integral at the various processing stages of the PDO trends depicted in Figure 2. PI was then regressed against cBc as shown in Figure 3. The curves showed a comparable intersection value (average PI equal to 12.9% at zero cBc), thus confirming the homogeneity of the fresh thighs. Moreover this result confirms that muscle protein degradation was initially driven by enzymes other than cB. Starting in the salting and resting phases the role of cB became important and remained active throughout the whole curing process [1]. However, Figure 3 shows that as the cumulative activity of cB increased, proteolysis increased, but at a different rate in the various PDOs as established by a parallelism test ($P \leq 0.05$). With an accelerated speed, at any level of cBc, proteolysis was greater in PA than in the saltier and drier TO hams. SA trend was intermediate, but more similar to PA hams. These curves suggest that different percentages of the potential cB activity, i.e. measured in the assay under optimal conditions, are effective on the muscle proteins during ripening in the three PDOs. A discrepancy between the "potential" and "actual" cB activity has recently been reported in Jinhua ham and assigned to the effect of the technological conditions, such as temperature, salt content and pH, on the enzyme activity [2]. This might also be the case in our conditions, given that salt content strongly

inhibited cB activity [2] and that we observed the lowest PI in the saltier TO ham. However, the addition of pepper and seasonings could also have further inhibited the enzyme activity during TO ripening. Moreover, an additional contribution related to a differential influence of the three technological conditions on the muscle protein accessibility to the endoprotease cannot be excluded, given that high salt also favours protein aggregation.

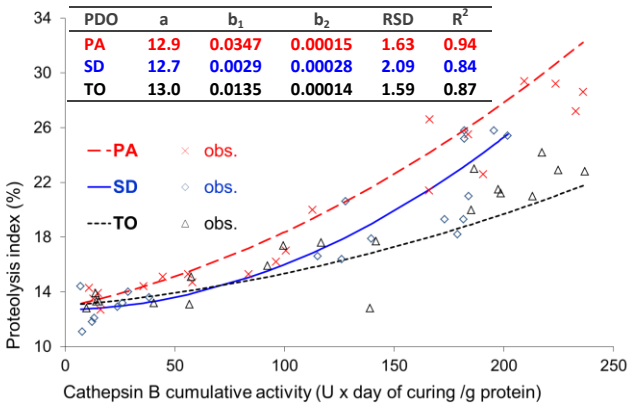

Figure 3. Relationship between PI and cBc of PDO hams at five processing phases: single observed values, and 2nd order regression lines, $PI = a+b_1\ cBc +b_2\ cBc^2$. In table: regression coefficients, residual standard deviation (RSD) and coefficient of determination (R^2)

The different technological conditions and intensity of proteolysis produced specific sensory properties in the various PDO hams, as demonstrated in the profile provided by trained assessors (data not reported). However, sensory defects such as bitter taste, mushy mouthfeel and pastiness detected in highly proteolysed PA hams [8], were not perceived, likely because cB did not reach the required, high thresholds of activity in the green thighs [3].

IV. CONCLUSION

The trend of cB activity during the ripening of the various PDOs was significantly different, particularly in the intermediate curing phases. This activity correlates with PI through a PDO dependent pattern. Indeed, at a same cumulative level of cB activity, the saltiest and driest TO ham, the only one with pepper and seasonings added, differed from the others by a lower PI. Then again, in PA ham, which is known to be more prone to defects of excess proteolysis, the degradation effect on muscular proteins was the highest, suggesting the existence of conditions contributing to a higher "actual" cB activity along with curing.

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