

A NEW METHOD AND INDEX FOR QUANTIFYING PROTEOLYSIS INTENSITY IN DRY-CURED HAM

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Abstract – A new method was developed to determine proteolysis activity in dry-cured ham using fluorescamine. A new proteolysis index (PI) was defined as the percentage ratio of the N-terminal α -amino group content to the total protein content of the ham extract. The robustness of the method was evaluated by measuring PI in laboratory pork meat samples subjected to standardized processing conditions and in samples extracted from industrial hams taken at different stages of processing. For the industrial samples, a comparison with the classic nitrogen procedure of PI determination was performed and a formula relating the two PIs was established. The rapidity, sensitivity and specificity of the procedure make it a good candidate for a screening test to evaluate ham quality in industry.

Key Words – dry-cured ham quality, fluorescamine, proteolysis index.

I. INTRODUCTION

In dry-cured ham, the main factor affecting final product quality is the proteolytic activity. It impacts the flavour and the texture [1-2]. Proteolytic activity depends on many factors, such as pH, water content, NaCl content and drying conditions [3]. In dry-cured ham, a proteolysis index (PI), defined as the percentage ratio of non-protein nitrogen content to total nitrogen content, is widely used to characterize the intensity of proteolytic activity; nitrogen content is determined by the Kjeldahl method [4]. Although this procedure is commonly used, it has many drawbacks. It is time- and product-consuming, and it lacks specificity, as there are many nitrogen compounds in meat (ammonium salts, creatinine, etc.) that can interfere in the determination of the proteolysis index. A more rapid and efficient assay of peptides and amino acids would thus greatly facilitate the evaluation of proteolysis in dry-cured ham. To this end, we

developed a simple, specific fluorometric procedure to determine the level of N-terminal α -amino groups of peptides and amino acids, which reflects the intensity of proteolytic activity during the curing process. Our procedure is based on the fluorescamine-specific labelling of the N-terminal α -amino groups present in the fractions of the ham extracts. A new proteolysis index was then defined as the percentage ratio of the N-terminal α -amino group content to the total protein content of the ham extract.

The new procedure was evaluated in small laboratory samples of pork meat processed under well-defined salting and drying conditions, and in industrial dry-cured hams at different stages of processing. For industrial dry-cured hams, a comparison of this new procedure with the commonly used nitrogen content procedure was performed, and a formula was established to convert the new proteolysis index to the classic one.

II. MATERIALS AND METHODS

Salted and dried pork meat samples were prepared at the laboratory in a way mimicking the different steps used in industry processing, but with adaptation to small samples. Three different muscles, *biceps femoris* (BF), *semi tendinosus* (ST), and *semi membranous* (SM), were used in the preparation of the laboratory samples. First, muscle surface was decontaminated using 0.1% peracetic acid. The muscle superficial layers, damaged by the acid treatment, were discarded, and samples were cut into small parallelepipeds (5×4×0.3 cm). These operations were performed under sterile air using sterile tools. The small pork meat samples were then salted by covering the surface of the piece with a 300 g.l⁻¹ NaCl solution using a

multichannel pipette (Multipette plus, Eppendorf AG, Hamburg, Germany). In these conditions, salt diffused in a manner to get in few hours a homogenous distribution. Drying was then performed at 15°C for different times until each sample reached the weight corresponding to the selected water content. Samples were then placed under vacuum in plastic bags and kept in temperature-controlled chambers (Model 14 D-78532, Binder GmbH, Tuttlingen, Germany) at different temperatures to obtain various proteolysis kinetics. At the end of the processing, samples were stored at -80°C until analysed. Four samples were prepared for each processing condition to make replicates.

Before analysis, the samples were placed in cold water to facilitate subsequent grinding. Extracts from the pork meat samples were prepared by homogenization with a Polytron PT-MR 2100 (Kinematica AG, Switzerland); aliquots of the extract were then removed and diluted with 12.5% trichloroacetic acid to precipitate proteins (TCA final concentration of 10%). Samples were shaken and centrifuged. The concentration of peptides and amino acids in the supernatant was measured by the method of Friguet et al. [5] with slight modifications. First, the supernatant was neutralized with 2 M potassium borate solution, pH 10. Second, fluorescamine at a concentration of 0.6 mg.ml⁻¹ in acetone was added. Fluorescence was measured 1 h after adding fluorescamine by means of a Perkin-Elmer LS 50B spectrofluorometer. A front surface accessory (Perkin Elmer Plate Reader) was installed for measurement in 96-well polystyrene microplates designed for fluorescence. Analyses were performed at the optimum excitation and emission wavelengths ($\lambda_{\text{excitation}} = 375 \text{ nm}$ and $\lambda_{\text{emission}} = 475 \text{ nm}$). The level of amino groups in the pork meat extracts was determined by reference to a calibration curve of glycine from 5 mM to 50 mM, treated in exactly the same conditions and at the same time as the pork meat extracts. Figure 1 shows that fluorescence increased linearly with glycine throughout the range of concentrations used here ($R^2 = 0.993$). The level of N-terminal α -amino groups was then expressed in grams of “glycine equivalent” per gram of ham (A). In parallel, the total protein content was estimated in the pork meat extract, before treatment, by the biuret

method. Results were expressed in grams of protein per gram of ham (B). The new proteolysis index was then expressed as the percentage ratio of N-terminal α -amino group content (A) to total protein content (B).

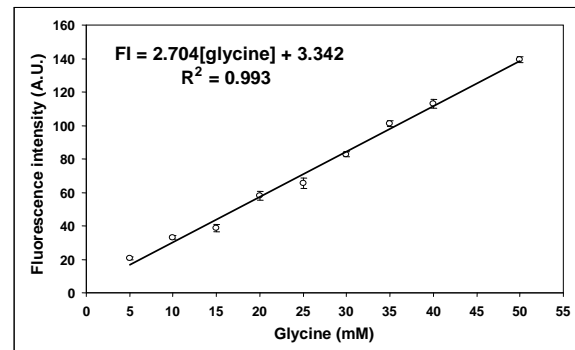


Figure 1. Calibration curve of glycine with fluorescamine

In parallel, PI determinations were performed on industrial samples taken from Bayonne dry-cured hams. Three muscles (*biceps femoris*, *semi tendinosus*, and *semi membranosus*) were extracted from three different hams at the end of each main processing stage, i.e. the resting period (11 weeks), drying period (21 weeks), and ageing period (52 weeks). Samples were taken from each muscle to calculate the new proteolysis index, allowing 24 determinations per muscle type per time (3 hams \times 8 samples). For the calculation of the classic proteolysis index, only one sample weighing about 50 g was taken from each muscle of the three hams. Total nitrogen (TN) was determined by the Kjeldahl method, and non-protein nitrogen (NPN) content by precipitation of proteins (TCA) followed by determination of nitrogen in the extract by the Kjeldahl method. The proteolysis index was determined as the percentage ratio of NPN to TN [6].

III. RESULTS AND DISCUSSION

The use of small pork meat samples enabled us to test a large number of conditions in muscles of a single animal to avoid animal effect and to shorten sample preparation time. The initial value of PI, measured in the *biceps femoris* muscle before processing, was very low

(1.04% \pm 0.33%). Figure 2 shows the time course of the PI measured in pork meat samples prepared from muscle *biceps femoris* and stored under vacuum for 12 days, at 25°C. The mean coefficient of variation of this parameter calculated from the whole conditions was 5.3%, demonstrating the good reproducibility of the procedure.

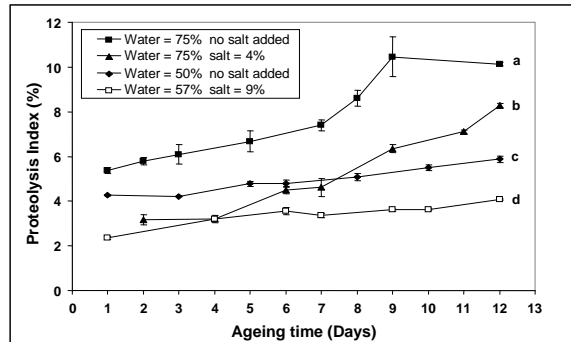


Figure 2. Proteolysis kinetics in *M. biceps femoris* at 25°C under different processing conditions (at the end of ageing, values not bearing common superscripts differ significantly, $p < 0.001$)

Figure 2 shows that the untreated samples (75% water and no salt added) exhibited the highest proteolysis index. Salting alone (to 4% of the dry matter) and drying alone (to 50% of water content) reduced the proteolysis intensity. This effect was much more pronounced when both salting and drying processes were applied (9% salt and 57% water). After 12 days of ageing, the four conditions tested exhibited significant differences in PI values among themselves ($p < 0.001$).

In the case of industrial dry-cured ham samples, to characterize the kinetics of proteolysis, the increase in PI with time was fitted by a second-order polynomial regression. Despite the biological variability, good correlation coefficients were obtained from the different curves ($R^2 > 0.99$). As shown in Figure 3, the three muscles tested here can be ranked in order of increasing proteolysis speed: BF > SM > ST. In the case of BF muscle, the proteolysis increased by 55% during the ageing period only. These results were in line with those of Zhao et al. [7], who reported a PI increase in BF muscle of 60% during the last 7 months of ageing.

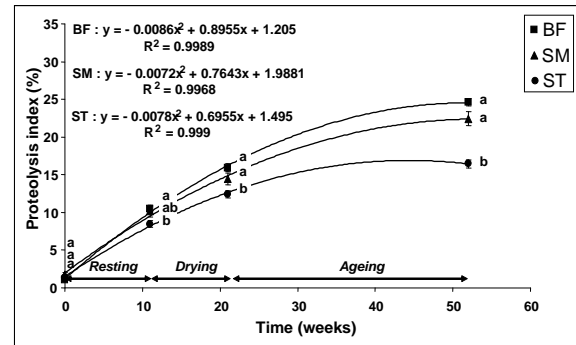


Figure 3. Proteolysis kinetics in three muscles of different Bayonne dry-cured hams. The zero time values corresponded to PI in the unsalted raw meats (values not bearing common superscripts differ significantly, $p < 0.05$)

We can attribute the higher proteolysis in the BF muscle to its higher residual moisture content, which allows a higher activity of endogenous proteases. Differences between SM and ST muscles could be explained by the fact that for similar water content, ST muscle exhibited higher salt content.

Hence although we have demonstrated the applicability of this new procedure in different conditions and its many advantages compared with the classic procedure, to be accepted by scientists and professionals in the dry-cured ham industry, the new proteolysis index must be easily convertible into the classic index. For this purpose, a conversion equation was established by comparison of the two indices in these same Bayonne hams at the end of the resting, drying and ageing periods.

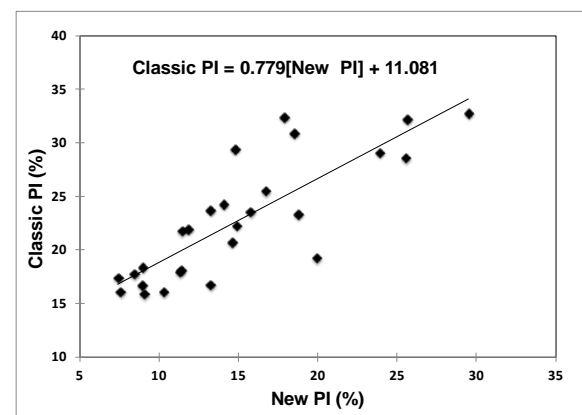


Figure 4. Relationship between the two proteolysis indices

Figure 4 shows the relationship between the two PIs, which is well fitted with a linear regression model: $PI_{\text{classic}} = 0.779 PI_{\text{new}} + 11.081$ ($r = 0.824$, $p < 0.001$).

This new procedure of PI determination offers many advantages over the classic nitrogen procedure. The fluorometric detection lends it a high sensitivity, which allows work on very small product quantities. Samples of 0.7 g were used in the present study, but we have successfully tested this procedure with smaller quantities of meat (less than 0.1 g, data not shown). Using this procedure, dry-cured hams could be tested during industrial fabrication by punching a small hole in the muscle, without depreciation of the products. In addition, the procedure is rapid; the limiting factor is the muscle extract preparation, but with trained personnel, more than 50 determinations can be performed daily. This procedure also offers a high specificity; fluorescamine does not react with the major non-protein nitrogenous substances commonly present in muscle, i.e. urea, uric acid, creatinine and ammonia. Also, fluorescamine does not react with secondary amines such as the amino groups of histidine and arginine side chains. Finally, it may be safely asserted that the new procedure is more powerful than the classic procedure in that it describes the progress of the proteolytic activity more finely.

IV. CONCLUSION

Based on these results, the fluorescamine-specific labelling of the N-terminal α -amino groups of peptides and amino acids offers a powerful technique to determine proteolysis intensity in dry-cured ham. In addition, we have developed a procedure for the easy detection of labelled amino groups in a fluorescence microplate reader. This study demonstrates some advantages of this procedure over the classic nitrogen procedure. It offers the possibility of rapidly determining peptides produced during the process on small amounts of sample. The easy conversion of PI values obtained with this new procedure into the classic PI values allows the fluorescamine method to be used directly in industry as a reference method for PI determination in dry-cured hams.

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