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Development of a rapid, specific and efficient procedure for the determination of proteolytic activity in dry-cured ham: Definition of a new proteolysis index

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ABSTRACT

A method was adapted to determine proteolytic activity in dry-cured ham using fluorescamine-specific labelling of N-terminal α-amino groups of peptides and amino acids. Fluorescence of the complex was measured using a microplate procedure and optimum excitation and emission wavelengths of 375 nm and 475 nm, respectively. 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 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.

1. Introduction

In dry-cured ham, one of the main factors affecting final product quality is proteolytic activity. It impacts the flavour, the appearance (e.g. tyrosine crystals, white film, brightness), and the texture (e.g. hardness, cohesiveness, and springiness) of the product (Arnau, Guerrero, & Sárraga, 1998; García-Garrido, Quiles-Zafra, Tapiador, & Luque de Castro, 2000; Toldrá & Flores, 2000; Virgili, Parolari, Schivazappa, Bordini, & Borri, 1995; Zhao et al., 2008). Proteolytic activity depends on many factors, such as pH, water content, NaCl content and drying conditions (Arnau, Gou, & Comaposada, 2003; Arnau, Gou, & Guerrero, 1994; Arnau, Guerrero, & Gou, 1997; Arnau, Guerrero, Maneja, & Gou, 1992; Martín, Córdoba, Antequera, Tímon, & Ventanas, 1998; Toldrá, Flores, & Sanz, 1997). 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, which measures ammonia after mineralization of the organic matter (Careri et al., 1993; Ruiz-Ramírez, Arnau, Serra, & Gou, 2006; Schivazappa et al., 2002). Although this procedure is commonly used and is standardized (ISO 937–1978 E reference method), it has many drawbacks. It is time- and product-consuming, and it lacks specificity, as there are many nitrogen compounds in meat (ammonium salts, urea, uric acid, 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, a simple, specific fluorometric procedure was developed 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. The procedure is based on the fluorescamine-specific labelling of the N-terminal α-amino groups present in the fractions of the ham extracts soluble in 10% trichloroacetic acid (TCA). 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. Because of the high sensitivity of fluorometric detection, fluorescamine has been extensively used for the quantification of amino acids, peptides and proteins (Bantan-Polak, Kassai, & Grant, 2001; Castell, Cervera, & Marco, 1979; Friguet, Stadtman, & Szewda, 1994; Lorenzen & Kennedy, 1993; Miedel, Hulmes, & Pan, 1989). In this study, this fluorophore was used to evaluate the proteolysis of skeletal muscle myofibrillar proteins (Morzel, Gatellier, Sayd, Renerre, & Laville, 2006).

The first objective of this study was to test the applicability of this procedure to the analysis of proteolysis in dry-cured ham at different stages of processing. For this purpose, determinations were performed in small laboratory samples of pork meat processed under well-defined salting and drying conditions, and in industrial dry-cured hams. The second objective was to compare this new procedure with the commonly used nitrogen content procedure and to establish an equation which allows converting the new proteolysis index to the classic one.

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2. Materials and methods

2.1. Preparation of the laboratory salted and dried pork meat samples

This preparation mimicked the different steps used in industry processing, but was adapted to small samples. Three different muscles, *biceps femoris* (BF), *semitendinosus* (ST), and *semimembranosus* (SM) from a single animal, were used in the preparation of the samples. pH_{24h} were 5.8 for BF, 5.7 for ST, and 5.6 for SM. First, muscle surfaces were decontaminated by using 0.1% peracetic acid for 3 min followed by a 1 min rinse with sterile water. This operation was repeated twice. 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) adjusted to 4 µl per spot. In these conditions, salt diffused so as to achieve a homogenous distribution in a few hours. The quantity of salt added was calculated on the basis of the salt concentration (4% to 13% of the dry matter) and water content (50% to 75%) required in the final pork meat samples. The percentages of salt obtained in the different samples were always contained between the expected percentage, by −1% to +1%, and the mean values calculated on four samples were close to the expected ones. Drying was then performed at 15 °C for different times (up to 16 h for the driest samples) until each sample reached the weight corresponding to the selected water content. After checking the water content by determining the dry matter and salt content by chlorometry (MKII Chloride Analyser 926, Sherwood, Cambridge, UK), samples were placed under vacuum in plastic bags and kept in temperature-controlled chambers (Model 14 D-78532, Binder GmbH, Tuttlingen, Germany) at different temperatures (4 °C, 14.5 °C and 25 °C) 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.

2.2. Preparation of the samples extracted from industrial dry-cured hams

PI determinations were performed on Bayonne dry-cured hams. Bayonne ham obtained a Protected Designation of Origin (PDO) certification in 1998 and its characteristics have been described by Robert and Lanore (2003). Three muscles (*biceps femoris*, *semitendinosus*, and *semimembranosus*) 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 (12 months). Samples were taken from each muscle to calculate the new proteolysis index, allowing 24 determinations per muscle type per time (3 hams×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. Some physical and chemical characteristics of these Bayonne hams (pH, water content and salt content) were determined (Table 1).

2.3. Determination of the new proteolysis index

Pork meat samples were placed for 1 h in cold water (0.7 g in 7 ml) to facilitate subsequent grinding. Extracts from the pork samples were prepared by homogenization with a Polytron PT-MR 2100 (Kinematica AG, Switzerland) for 30 s at maximum speed (30,000 rpm); 150 µl aliquots of the extract were then removed and diluted with 600 µl of 12.5% trichloroacetic acid to precipitate proteins (TCA final concentration 10%). Samples were shaken for 15 min at 4 °C. After centrifuging at 2000 g for 10 min, the concentration of peptides and amino acids in the supernatant was measured by the method of Friguet et al. (1994) with slight modifications. First, 300 µl of the supernatant was neutralized with 300 µl of 2 M sodium borate, pH 10. In these conditions, the final pH of the solution was 9.2 +/− 0.1, a value suitable for the reaction with fluorescamine. Secondly, 180 µl of fluorescamine (Sigma) at a concentration of 0.6 $mg.ml^{-1}$ in acetone was added. Free fluorescamine does not fluoresce, but its reaction with primary amines yields a fluorescent pyrolinone moiety. 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. A volume of 200 µl was placed in each well. Analyses were performed at the optimum excitation and emission wavelengths ($\lambda_{excitation}$ = 375 nm and $\lambda_{emission}$ = 475 nm) with excitation and emission slit setting to 10 nm. The non-specific fluorescence of corresponding fluorescamine-untreated samples was subtracted. 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 (concentration of the stock solution before TCA treatment) treated under exactly the same conditions and at the same time as the pork meat extracts. Fig. 1 shows that fluorescence increased linearly with glycine throughout the range of concentrations used (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 (Gornall, Bardawill, & David, 1949). Results were expressed in grams of protein per gram of ham (B). The new proteolysis index was then expressed as the percentage

Table 1
Characteristics of the Bayonne dry-cured hams at the end of each main processing stage. Water and NaCl contents were calculated on the basis of the total and dry matter, respectively. Values were the means +/− SEM of 3 determinations (one determination per muscle). Values not bearing common superscripts differ significantly (p<0.05).

		pH	Water content (%)	NaCl content (%)
11 weeks	SM	5.85 +/− 0.05 a	69.8 +/− 0.6 ab	2.67 +/− 0.42 ab
	ST	5.95 +/− 0.09 a	68.0 +/− 0.1 a	4.63 +/− 0.13 a
	BF	5.90 +/− 0.04 a	71.6 +/− 0.2 b	2.39 +/− 0.28 b
21 weeks	SM	5.88 +/− 0.11 a	61.1 +/− 1.2 a	3.38 +/− 0.47 a
	ST	5.87 +/− 0.11 a	65.3 +/− 1.0 ab	3.40 +/− 0.54 a
	BF	5.95 +/− 0.13 a	68.2 +/− 0.5 b	3.72 +/− 0.59 a
12 months	SM	5.68 +/− 0.05 a	56.4 +/− 1.0 a	4.94 +/− 0.22 a
	ST	5.78 +/− 0.03 a	57.2 +/− 1.4 a	5.41 +/− 0.26 a
	BF	5.83 +/− 0.03 a	60.5 +/− 0.7 a	5.72 +/− 0.33 a

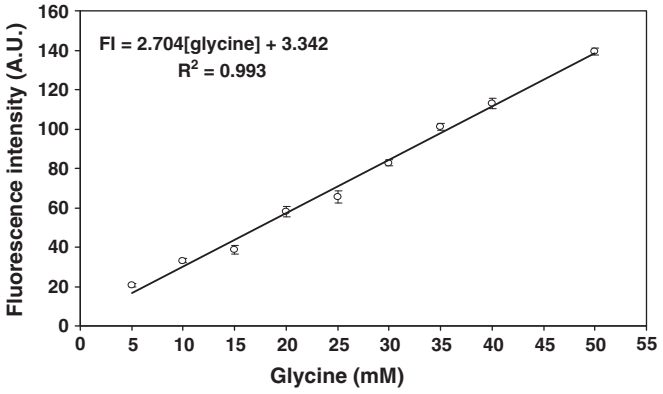

Fig. 1. Calibration curve of glycine with fluorescamine. Glycine concentrations were those of the initial stock solutions before the TCA treatment. Values were the means +/− SEM of four independent determinations.