Green ham pH value affects proteomic profile of dry-cured ham

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SHORT COMMUNICATION

Green ham pH value affects proteomic profile of dry-cured ham

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Abstract

In the present study we investigated the effect of green ham pH value on the proteomic profile of *m. biceps femoris* of the 14 month old '*Kraški pršut*' dry hams. Two groups (n=12) of samples were chosen according to green ham *m. semimembranosus* pH (*i.e.* low pH group with values from 5.51 to 5.60 and high pH group with values from 5.80 to 6.18). Two groups of hams were similar with regard to fat thickness and ham weight. The myofibrillar muscle protein fraction was extracted from dry-cured ham *m. biceps femoris* and separated using 2-dimensional electrophoresis technique. More than 1,000 protein spots were detected on the gels, out of which 100 spots had significantly different intensity according to pH group. Notable clustering of the spots was observed on the gel images. Namely, the protein spots differentiating low and high pH groups were more intense in the acidic part of the gel for the low pH group, and in the basic part for the high pH group. The proteomic approach proved to be a suitable tool to investigate the influence of green ham pH on the pattern of protein degradation. However, further research (protein spot identification, association with sensory properties) is in progress.

Introduction

In recent years, the use of proteomic tools has been expanded to the field of meat quality (Hollung *et al.,* 2007). Two-dimensional electrophoresis (2DE) based proteome analysis allows a large number of proteins (more than 1,000 in one gel) to be characterized at the same time. Thus it represents a valuable tool for the study of complex traits and identification of molecular markers of food quality. Over the last years, several studies of proteomic research in meat science have been conducted (for review see Hollung *et al.,* 2007). However the studies related to dry-cured ham are rare (Di Luccia *et al.,* 2005; Singh Sidhu *et al.,* 2005). Proteolysis of muscle proteins, resulting from the action of endogenous enzymes, is one of the key biochemical reactions taking place during the processing of dry-cured ham, affecting also its sensory quality (Toldra and Flores, 1998). The degree of proteolysis depends on raw material properties (ham weight, pH, fatness, water binding capacity) and processing conditions (determining salt penetration and water availability) which affect the activity of the enzymes (for review see Buscailhon and Monin, 1994). The pH value is considered a fundamental parameter for pork quality, and has been shown to affect the proteolysis in dry hams (Arnau *et al.,* 1998; Schivazappa *et al.,* 2002). It has a direct action on enzyme activity and an indirect impact through its action on water loss and salt penetration (Toldra, 2002). Lower green ham pH has been associated with higher cathepsin activity and higher non-protein nitrogen (Arnau *et al.,* 1998; Schivazappa *et al.,* 2002). On the other hand, an intense liquid exudation of low pH hams encourages salt solubilization and absorption (Arnau *et al.,* 1995) and thus higher salt intake (Garcia-Rey *et al.,* 2004; Ruiz-Ramirez *et al.,* 2006), therefore inducing just the opposite (inhibitory) effect on the proteolytic activity. The present work presents preliminary results of the proteomic profile of insoluble (myofibrillar) fraction of dry-cured ham *m. biceps femoris* in relation to green ham pH value.

Materials and methods

Ham collection

Material included in the present experiment originated from an extensive study on dry hams performed within the EU Truefood project (Škrlep *et al.,* 2008; Santé-Lhoutellier *et al.,* 2009). Hams were obtained from approximately six month old commercial pig fatteners of one genotype (crosses of Large White × Landrace as maternal and Duroc × Hampshire as paternal line). Hams were processed according to the rules of Slovenian dry ham *"Kraški pršut"* (green ham weight above 9.5 kg, subcutaneous fat thickness above 10 mm, and non-PSE or non-DFD appearance). Processing consisted of two salting phases, resting phase, drying phase and maturation phase (in total 60 weeks). Prior to salting (two days after slaughter) green ham weight, ham fat thickness (below *ossis caput femoris*), pH value in *m. semimembranosus* (SM) using MP120 pH meter (Mettler Toledo, Schwarzenbach, Switzerland) and Minolta L*a*b color values (Minolta Chroma Meter CR-300, Minolta Co, Osaka, Japan) of *m. gluteus medius* (GM) were measured. The pH value represents an average of the measurements on two locations in the muscle (first on the inner edge of SM, adjacent to the *caput ossis femoris* and the second on the caudal edge of the open surface of the muscle) to take into account muscle heterogeneity. A subsample of 12 hams was chosen for proteomic analysis based on SM pH values, *i.e.* 6 hams of the lower quartile pH group (5.51-5.60) and 6 hams of the higher quartile pH group (5.80-6.18), also taking into account the processing batch (2 hams per batch).

Sample preparation

For the proteomic analysis, a 5 mm thick slice of BF was taken from the central part of dry hams. The samples were frozen in liquid nitrogen and ground to fine dust using a labo-

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ratory mill (IKA M120, IKA Werke, Staufen, Germany). Protein extraction and solubilization were performed according to the method developed at the INRA. Briefly, insoluble protein fraction was extracted with repeated washing in low ionic strength buffer and the remaining pellet stored at -80°C until use. Protein concentration was determined using the Bradford protein assay (Bio-Rad, CA, USA) and the samples were diluted 1:50 with water prior to the measurement.

Two-dimensional electrophoresis

Prior to the electrophoresis, 1000 μg of protein samples were diluted with rehydration buffer (containing 7M urea, 2M thiourea, 2% CHAPS, 0.0002% bromphenol blue and 0.5% carrier ampholytes), loaded on immobilized pH gradient (IPG) strips (Bio-Rad ReadyStrip, 17 cm, pH 3-10 non-linear), covered with mineral oil and left to rehydrate for 16 hours in a dry strip reswelling tray. The next day, isoelectric focusing (IEF) was carried out on the Ettan IGPhor 3 IEF unit (GE Healthcare Bio-Sciences AB, Uppsala, Sweden). Low voltage (50 V) was applied in the initial step followed by a stepwise increase to 5,000 V, reaching a total of 70,000 Vh (adapted from the INRA protocol).

Prior to SDS-PAGE, strips were equilibrated in equilibration solution containing 50 mM Tris-HCl buffer, pH 8.8, containing 6 M urea, 30% (v/v) glycerol, 2% (w/v) SDS and 1% (w/v) DTT for 15 min, followed by additional incubation for 15 min with the same buffer replacing DTT with 2.5% (w/v) iodoacetamide. SDS-PAGE was performed on 12.5% polyacrylamide gels using Ettan DaltSix (GE Healthcare Bio-Sciences AB, Uppsala, Sweden) at 10 mA/gel for one hour and afterwards at 40 mA/gel until the dye front reached the end of the gels. For the assessment of molecular weight (MW), protein MW marker #SM0431 (Fermentas Life Sciences, Glen Burnie, MD, USA) was applied prior to running the second dimension. The gels were stained with Coomassie Brilliant Blue G250 in 3% (v/v) phosphoric acid, 17% (w/v) ammonium sulphate and 34% (v/v) methanol for 96 hours and washed with water. The gel images were digitalized by Image Scanner III (GE Healthcare Bio-Sciences AB, Uppsala, Sweden). For each sample, three technical repetitions (resulting in 36 gels) were made.

Image and data analysis

Images were analyzed using ImageMaster 2D Platinum 6 software (GE Healthcare Bio-Sciences AB, Uppsala, Sweden). Following spot detection, the gel images were automatically matched to the master gel. Relative spot volumes (%vol) were calculated as a ratio between the volume of the individual spot and the volume of all the spots on the gel to account for the variations due to protein loading and staining. The values were then log-transformed as suggested by Meleth *et al.* (2005). Indefinable or over-expressed spots were not included in the analysis. Only the spots present in at least two technical repetitions and in all biological repetitions (hams) were taken. Relative spot volume value per ham corresponds to the mean value of its technical repetitions. The data were exported to SAS software (SAS Inc., Cary, NC, USA) in order to perform analysis of variance with fixed effect of pH group (GLM procedure).

Results and discussion

Green ham properties of the low or high pH group are presented in Table 1 showing that the two groups of hams differed only in pH value while they were similar in relation to weight and fatness, also known to influence

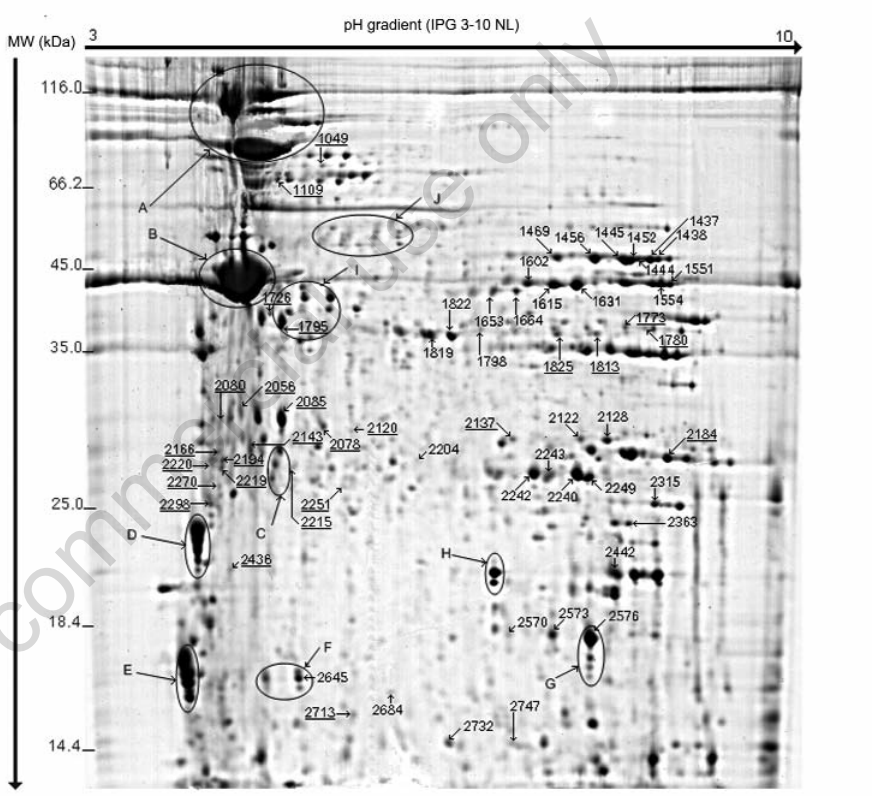

Figure 1. Scanned 2DE image of insoluble protein fraction of *m. biceps femoris* separated by pH using a 3-10 non linear (NL) immobilized pH gradient (IPG) strip in the first dimension and molecular weight (MW) on 12.5% polyacrylamide gel in the second dimension. Numbered spots are presented in detail in Table 2.

Table 1. Green ham properties (mean ± SE) according to pH group.

	High pH	Low pH	Significance (P)
Number of hams	6	6	
GM L*	44.01±1.61	50.92±1.04	0.0049
GM a*	9.87±0.45	10.07±0.35	0.7308
GM b*	3.06±0.21	5.66±0.51	0.0009
SM pH	5.97±0.06	5.55±0.01	< 0.0001
Ham fat thickness, mm	14.4±1.4	13.8±0.7	0.7306
Ham weight, kg	10.73±0.29	10.79±0.23	0.8792

GM: *gluteus medius* muscle; SM: *semimembranosus* muscle.

dry ham seasoning ability and quality (Russo and Nanni Costa, 1995; Bosi and Russo, 2004). It is also worth mentioning that the hams were selected against PSE and DFD appearance (consortium rules), therefore excluding the most extreme pH values. In accordance with ham pH group, along with pH differences, Minolta color parameters differed between the two groups (higher L* and b* denoting lighter color with higher yellow tone were observed in the low pH group). Although pH and color were measured in different muscles (SM and GM, respectively), the correlations were relatively high (-0.72 with Minolta L and -0.84 with Minolta b*; *data not shown*), demonstrating that both muscles can serve as indicators of ham quality. Namely, BF is inaccessible on green ham at the moment of salting and measurements cannot be taken on this muscle. However, quality parameters of SM, as a surface muscle, determine water loss and salt intake dynamics, therefore they are expected to also have an effect on BF. The decision to study the proteolysis in BF derives from the fact that problems with texture and sensory quality due to increased proteolysis were mostly reported for this muscle (Parolari *et al.,* 1994; Arnau *et al.,* 1998; Garcia-Garrido *et al.,* 1999; Morales *et al.,* 2007). As an internal muscle, the BF muscle is not directly exposed to the salted surface and the salt reaches it at the later stages of processing (Toldra, 2002; Fantazzini *et al.,* 2009). Consequently, high water activity and content accompanied by lower salt concentration in the initial stages of processing imply a higher proteolysis activity in BF muscle. According to the literature (Guerrero *et al.,* 1999; Schivazappa *et al.,* 2002), lower green ham pH is expected to enhance proteolysis in BF muscle, which we wanted to observe at the proteomic level.

Figure 1 shows a representative 2DE gel image of insoluble muscle protein fraction of dry-cured ham (BF) aged for 14 months. On the upper left quadrant of the gel (higher molecular weight (MW), acidic side), there are several very distinctive spots, which according to the available literature for fresh pig muscles (Lametsch *et al.,* 2003; Morzel *et al.,* 2004; Hwang *et al.,* 2005; Laville *et al.,* 2005), could belong to the actomyosin complex, the most abundant protein fraction in the skeletal muscle. The groups of protein spots on the gel image marked with letters A and B were very strongly expressed and unclear on all of the gels. Due to this overexpression, these spots could not be analyzed; however, they merit further investigation (e.g. using IPG strips with a narrower pH range). It is worth mentioning the observed difference in the pattern of spots of the present study compared to the pattern of spots in the corresponding regions reported for the fresh pig muscle (Lametsch *et al.,* 2003; Morzel *et al.,* 2004; Hwang *et al.,* 2005; Laville *et al.,* 2005). Namely, in the present study we observed vertical chains of spots as denoted on the Figure 1 (C, D, E, F, G, H, I, J). It would be interesting to verify if these spot chains consist of a degradation of the proteins to lower MW fragments during the ripening. However, a direct comparison to the situation in green ham is not possible without protein identification, which will be a subject of our further studies. For the moment, our basic aim was to show spot manifestation according to pH value of green ham muscle, known for its impact on dry ham quality.

In the present study, a total of 1,187 different protein spots were determined on the gels, which can be considered as a normal quantity of spots in the given conditions (Hollung *et al.,* 2007). Due to the missing values and elimination of highly saturated or ill defined spots, 932 spots were finally included in the statistical analysis for the pH effect. Out of this spot pool, 100 spots demonstrated significantly different intensity (%vol) according to the pH group (*data not shown*) which confirms the importance of pH for muscle proteolysis. In Table 2 and Figure 1 only the clearest and most significant spots (n= 60) are shown and discussed further. It could be seen that the protein spots differentiating the low and high pH groups were more intense in the acidic part of the gel for the low pH group (spot id numbers underlined), and in the basic part of the gel for the high pH group. Since no parallel control analysis on the green ham was carried out, we can speculate that the difference in spot volume is a consequence of differential activity of proteolytic enzymes due to the pH condition; however, we cannot exclude the initial differences in protein expression. While lower pH is expected to increase cathepsin activity (Arnau *et al.,* 1998; Schivazappa *et al.,* 2002) and consequently the extent of proteolysis, a low pH is also expected to increase salt intake (Garcia-Rey *et al.,* 2004; Ruiz-Ramirez *et al.,* 2006) which has an inhibitory action on proteolytic enzymes. Moreover, a differential pattern of protein degradation in relation to pH value can also be explained by the fact that not all enzymes are equally inhibited by increasing salt concentration during dry ham ripening. For instance, the activity of several tested peptidases and cathepsin D is largely reduced (75-90%) in the presence of 5% NaCl, while the activity of cathepsin L is reduced by only 10%, and the activity of aminopeptidase B is even enhanced (Toldra and Flores, 1998).

Table 2. Relative spot volume (%vol) according to pH group.

		Mean %vol		
Spot	Fold	High pH	Low pH	P
1049	1.4	0.02033	0.02881	0.008
1109	1.4	0.03315	0.04583	0.040
1437	2.0	0.09600	0.04828	0.012
1438	2.2	0.09566	0.04429	0.009
1444	1.5	0.06819	0.04538	0.009
1445	1.9	0.27806	0.14996	0.005
1452	2.3	0.18818	0.08302	0.005
1456	1.9	0.28745	0.14979	0.002
1469	1.5	0.12815	0.08273	0.001
1551	1.6	0.15876	0.09752	0.015
1554	1.5	0.18370	0.12015	0.020
1602	1.9	0.18846	0.10120	0.004
1615	2.1	0.28036	0.13320	0.013
1631	2.0	0.38945	0.19389	0.036
1653	1.6	0.09622	0.06021	0.008
1664	2.0	0.06553	0.03307	0.010
1726	1.5	0.02248	0.03455	0.030
1773	1.4	0.04851	0.06838	0.027
1780	1.7	0.03482	0.05804	0.000
1795	2.7	0.04290	0.11787	0.022
1798	1.5	0.02847	0.01962	0.015
1813	1.5	0.03399	0.05128	0.046
1819	1.3	0.06255	0.04662	0.034
1822	1.9	0.07803	0.04050	0.002
1825	1.3	0.04373	0.05489	0.020
2056	1.6	0.11046	0.18140	0.015
2078	1.7	0.07619	0.12589	0.008
2080	2.0	0.04151	0.08202	0.035
2085	1.4	0.22183	0.31030	0.027
2120	1.6	0.02354	0.03708	0.001
2122	1.5	0.08168	0.05621	0.005
2128	2.0	0.08220	0.04182	0.003
2137	1.6	0.02087	0.03419	0.007
2143	1.5	0.07714	0.11495	0.045
2166	2.8	0.02022	0.05696	0.016
2184	1.7	0.06005	0.09937	0.037
2194	1.6	0.03347	0.05436	0.047
2204	2.2	0.03794	0.01709	0.001
2215	2.5	0.02123	0.05307	0.004
2219	3.1	0.02475	0.07760	0.002
2220	3.6	0.02470	0.08930	0.044
2240	3.0	0.46074	0.15118	0.003
2242	1.6	0.24540	0.15080	0.001
2243	1.6	0.16710	0.10596	0.011
2249	2.2	0.16302	0.07553	0.004
2251	1.5	0.02597	0.03936	0.039
2270	2.3	0.01165	0.02710	0.041
2298	1.7	0.12316	0.20324	0.032
2315	1.7	0.07046	0.04219	0.027
2363	1.8	0.07415	0.04194	0.006
2436	1.6	0.03491	0.05655	0.038
2442	1.4	0.27884	0.20597	0.004
2570	1.6	0.06815	0.04219	0.010
2573	1.4	0.14906	0.10310	0.042
2576	2.2	0.52416	0.23399	0.002
2645	1.6	0.08133	0.05110	0.001
2684	1.7	0.02924	0.01706	0.037
2713	1.4	0.05355	0.07376	0.020
2732	1.6	0.04917	0.03080	0.000
2747	1.5	0.05649	0.03725	0.021

Fold is a quotient between spot intensities (% vol) of two groups. P: level of significance.

Conclusions

The results of this preliminary study demonstrate a notable effect of green ham pH on the proteomic profile of dry cured ham BF muscle. Additional studies (e.g. protein identification) are needed to further substantiate our results. A proteomic approach offers a new and different insight into the proteolysis occurring in dry ham and merits further investigation as a tool for the identification of protein markers of dry-cured ham quality.

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