JOURNAL OF
AGRICULTURAL AND
FOOD CHEMISTRY

Effect of Extended Aging of Parma Dry-Cured Ham on the Content of Oligopeptides and Free Amino Acids

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The effect of the dry-curing processing time on the release of oligopeptides and amino acids was evaluated with 158 Parma hams subdivided into three groups: (1) traditional processing (450 days); (2) extended processing (570 days); and (3) extended aging (690 days). Most of the oligopeptides and free amino acids detected increased up to the last deadline (690 days); a sharp increase of peptides below 400 Da was the main change in most aged hams. In particular, γ-glutamyl dipeptides showed a remarkable increase during ham extended aging, acting like permanent taste-active compounds, being unsuitable for further enzymatic breakdown. The pH of fresh hams showed negative relationships ($P < 0.001$) with most peptides. With regard to free amino acids, the pattern was modified by different processing lengths, together with their taste categories, so that the amino acids having monosodium glutamate-like and bitter tastes were enhanced in more aged hams.

KEYWORDS: Dry-cured ham; aging time; oligopeptides; γ-glutamyl dipeptides; free amino acids

INTRODUCTION

The flavor of dry-cured ham relies on a blend of volatile and nonvolatile molecules resulting from many reactions that take place during dry-cured ham processing. As far as the volatile compounds are concerned, the positive role in the enhancement of aroma played by aldehydes and ketones, derived from lipid hydrolysis and autoxidation, and by ethyl and methyl esters, derived from the degradation of branched chain amino acids (BCAA), was demonstrated for several types of dry-cured hams (*1*−*3*). Nonvolatile molecules (peptides and free amino acids), mainly released by the activity of endogenous proteolytic enzymes, are also involved in the characteristic ham taste and flavor development. The formation of peptides during Parma ham processing was highly correlated to the flavor formation, and in particular the hydrophobic peptides were associated with a bitterness perception (*4*, *5*).

Data on the identification of specific peptide sequences directly related to the ham sensory quality are quite scarce in the literature. Some authors agree on the role played by dipeptides containing hydrophobic amino acids (phenylalanine, leucine, isoleucine, valine, tryptophan) in enhancing the perception of the bitter taste in dry-cured ham. On the other hand, the dipeptide Glu−Tyr has been related to aged taste, and the dipeptides Val−Glu and Gly−Glu have been related to sour taste (*6*, *7*).

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Amino acids are known to have a great impact on the flavor of a variety of foods. It is well-known that a large increase of amino acids occurs during the processing of dry-cured ham, and it is continual during proper aging (*8*). However, an excessive amount of free amino acids seems to be related to the onset of unpleasant bitter and sour taste, also because the amino acids showing the greatest increase in more proteolyzed dry-cured hams are known as having a bitter flavor (*6*, *9*). Aspartic and glutamic acids were related to the "umami" taste, whereas hydrophobic amino acids were highly related to bitterness (*6*, *9*); in the case of phenylalanine, isoleucine, and leucine, the positive correlation found with the bitterness is consistent with their low bitterness threshold values (*10*).

Several attempts were made to improve the quality standard of dry-cured hams by selecting a more suitable raw meat for dry-curing, by lowering the salt content, and by controlling the process temperature and time. Although these changes yielded better products in terms of sensory, nutritional, and safety properties, the actual molecules responsible for the dry-cured ham quality have not yet been identified or are still a matter of discussion. Most authors agree with the opinion that the bulk increase in proteolysis products does not mean necessarily a quality improvement; therefore, it seems more promising to investigate which specific proteolytic patterns or products are most suitable for enhancing dry-cured ham taste.

The aim of this work is to investigate the development of oligopeptides and free amino acids in Parma dry-cured hams processed for different times. Because dry-cured hams are reported to achieve a better flavor after a protracted processing

10.1021/jf061312+ CCC: $33.50 © 2006 American Chemical Society
Published on Web 11/07/2006

time (*8*, *11*), it would be very important to identify sound indices of flavor quality in dry-cured hams.

MATERIALS AND METHODS

One hundred and fifty-eight trimmed raw hams from breed crosses, slaughter age, and weight range (13.5−14.5 kg) representative of domestic heavy pigs used in the production of typical dry-cured ham in Italy were purchased from one slaughterhouse. To take into account the variability usually occurring both in raw meat (breed cross, genotype, breeding and feeding procedures, slaughtering day) and in dry-cured ham manufacturing, the fresh hams were taken from four numerically homogeneous batches of pigs coming from different breeding houses and slaughtering days and delivered to two plants operating according to the guidelines of the Parma ham consortium, that is, mandatory basic regulations for protected designation of origin (POD) dry-cured ham manufacturing (*12*).

pH of Fresh Hams. The pH in the semimembranosus muscle was measured at 24 h post-mortem (pH_{24h}) with a Hamilton glass electrode probe attached to a portable pH-meter (WTW pH330, Weilheim, Germany).

Ham Processing and Sampling. Raw hams from each batch were subgrouped to be processed for different times in subsequent manufacturing sessions held in both plants. During the first curing step all hams were processed in the same way, then one group ($n = 48$) was processed up to 450 days (traditional processing), another ($n = 56$) up to 570 days (extended processing), and another ($n = 54$) up to 690 days (extended aging). Samples for analyses (biceps femoris muscle) were taken at each established stage. The biceps femoris muscles were cut from the center of each ham from the cushion area, perpendicular to the femur bone; only intramuscular fat was included in the samples. After pH measurement ($pH_{dry-cured}$) by inserting the glass electrode perpendicular to the biceps femoris muscle section, the samples were stored in vacuum-sealed bags and stored at 2 °C until analyzed (within 10 days).

Moisture was determined as the weight loss of ca. 2 g of minced muscle after drying at 100−102 °C for 16−18 h according to the AOAC method (*13*).

Proteins were determined as total nitrogen in ca. 2 g of minced muscle by using the Kjeldahl method and calculated as N × 6.25 following the AOAC method (*13*).

Salt content was estimated in 10 g of minced muscle as chlorides, which were extracted with hot water (40 °C) and quantified by using the Carpentier−Volhard method, according to the AOAC method (*13*).

Proteolysis index was measured according to the method of ref *2* and expressed as percent ratio between the nitrogen soluble in 5% trichloroacetic acid and the total nitrogen.

Free amino acids were analyzed by HPLC (*14*); the separation was performed using an Agilent 1100 (Boeblingen, Germany) and a Hypersil ODS column 200 × 2.1 mm (Agilent Technologies) supported with a Hypersil ODS guard column 20 × 2.1 mm (Agilent Technologies). Primary amino acids were detected as OPA derivatives at 338 nm (multiple-wavelength diode array detector, Agilent Technologies 1100) and secondary amino acids as FMOC derivatives at 226 nm (excitation) and 313 nm (emission) with a fluorescence detector (Agilent Technologies 1100). Data acquisition was accomplished by the Agilent ChemStation software. For amino acid identificatin, solutions of standard amino acids were used.

Oligopeptides. A total of 10 g of sample was homogenized with an Ultra Turrax T50 (Janke and Hunkel Italabortechnik) with 90 mL of 0.1 N HCl and 0.49 mL of 1 mM (L,L)-phenylalanylphenylalanine (Phe−Phe, internal standard). The homogenate was centrifuged at 13500*g* for 20 min at 5 °C. The solution was subsequently filtered through glass wool, a paper filter (pore dimension = 15−20 μm), and 5 μm filters. The resulting liquid phase was extracted three times with 20 mL of ethyl ether. The solution was filtered again with a Millipore 46 mm Steril Aseptic System through 0.45 μm HVLP Millipore filters. A total of 4 mL of the filtrate was evaporated to dryness, dissolved in 2 mL of a formic acid solution (pH 3), and then diafiltered using an Amicon Micropartition system MPS-1. The solution was diafiltered through Amicon Ultrafilters YM10 filters (nominal molecular cutoff of 10000 Da) and then through Amicon Ultrafilters YM3 (nominal molecular cutoff of 3000 Da). The filtrate was dried under nitrogen and dissolved in 200 μL of an aqueous solution containing 10% CH_3CN and 0.1% HCOOH.

Table 1. Effect of Aging Time on pH_{24h}, $pH_{dry-cured\ ham}$, Moisture, Protein, Salt, and Proteolysis Index of Parma Dry-Cured Hams[a]

	processing time		
variable	450 days ($n = 48$)	570 days ($n = 56$)	690 days ($n = 54$)
pH_{24h}[b]	5.73 ± 0.15	5.74 ± 0.14	5.73 ± 0.12
$pH_{dry-cured\ ham}$[c]	5.88 ± 0.11	5.91 ± 0.09	5.89 ± 0.12
moisture[d]	61.5 ± 1.2 a	60.0 ± 1.2 b	58.3 ± 1.5 c
protein[d]	27.4 ± 1.1 c	28.7 ± 1.1 b	29.9 ± 1.1 a
salt (NaCl)[d]	4.79 ± 0.54 b	5.12 ± 0.54 a	5.32 ± 0.48 a
proteolysis index[e]	30.4 ± 1.7 c	31.8 ± 2.0 b	32.9 ± 2.1 a

[a] Estimated means within a row with different lower case letters are different ($P < 0.05$). [b] Measured at 24 h post-mortem in the semimembranosus muscle. [c] Measured at the end of each assayed processing time in the biceps femoris muscle. [d] Expressed in grams per 100 g of biceps femoris muscle. [e] Expressed as percent ratio between the nitrogen soluble in 5% trichloroacetic acid and the total nitrogen.

The fractions thus obtained were analyzed by HPLC-MS (Water Alliance 2695 separation module connected with a Micromass ZMD mass spectrometer). The separation was performed with a Jupiter (Phenomenex) C18 column (300 Å, 250 × 4.6 mm) using an elution gradient: eluent A, H_2O (0.2% CH_3CN, 0.1% HCOOH); eluent B, H_2O/CH_3CN 65:35 (0.1% HCOOH); elution, 0−15 min isocratic 99% A, 15−69 min linear gradient from 99 to 15% A, 60−82 min isocratic 15% A, 82−83 min from 15 to 99% A; flow rate, 1 mL/min. MS conditions: capillary voltage, 3 kV; cone voltage, 30 V; positive ion mode; source temperature, 100 °C; desolvation temperature, 150 °C; cone gas (N_2), 100 L/h; desolvation gas (N_2), 400 L/h. Oligopeptide analysis was carried out on 72 dry-cured hams, selected to be representative of different batches, manufacturing plants, and processing times.

Statistical Analysis. Data were analyzed as a randomized incomplete unblocked design with the General Linear Model procedures of SPSS (SPSS/PC, ver. 12, 2003). The model for the analysis of all variables included the fixed effects of processing time, manufacturing plant, and curing batch and the covariate fresh ham pH (pH_{24h}). The pooled error term was used to test the main effects, least-squares means were computed for one main effect (processing time), and the Bonferroni *t* test was used to statistically separate least-squares means when $P < 0.05$. One-way analysis of variance was run to compare composition, proteolysis, and pH of dry-cured hams corresponding to different processing times, and the Bonferroni *t* test was used to statistically separate least-squares means when $P < 0.05$.

RESULTS AND DISCUSSION

Dry-Cured Ham Composition. The extended processing time induced differences ($P < 0.05$) in dry-cured ham composition (biceps femoris muscle) in terms of moisture, salt, proteins, and proteolysis index (**Table 1**), the changes of the chemical components being due to the increased dehydration occurring during the extended aging. Average pH values were similar among groups, for both fresh and aged hams.

Free amino acids and di- and tripeptides are the molecules mainly contributing to the proteolysis index because it has been demonstrated that the deproteinizing agent trichloroacetic acid precipitates the peptides with more than three or four residues (*15*). Because free amino acids and short peptides are the main final products of the proteolytic process, it is not surprising to observe the significant increase of this index with protracted aging, as agreed upon by several authors (*11*, *16*). Therefore, the proteolysis index may be regarded as a bulk proteolysis

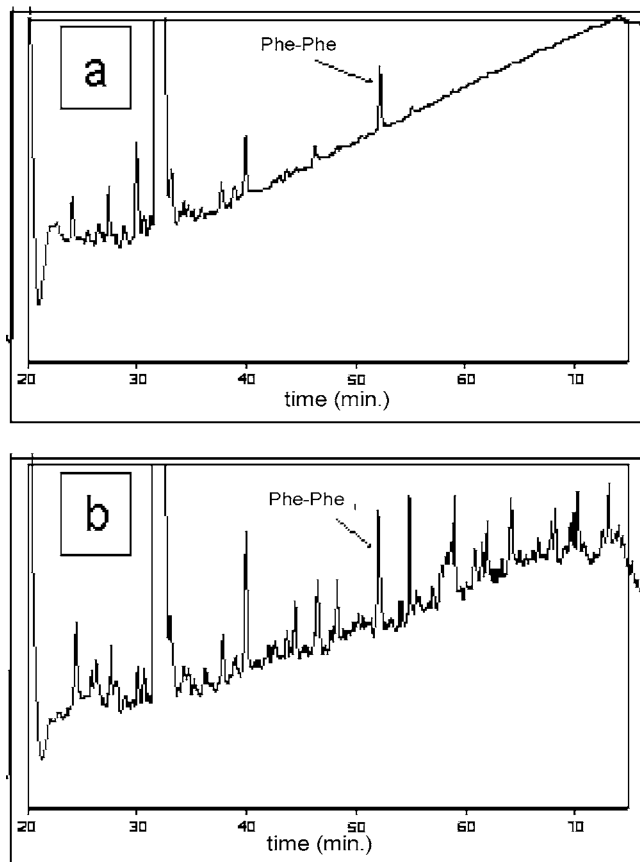

Figure 1. HPLC analyses of Parma dry-cured ham oligopeptide fraction obtained with two different extraction methods (detection at $\lambda = 214$ nm): (**a**) chromatogram of the fraction obtained using 0.1 N HCl followed by 5% trichloroacetic acid; (**b**) chromatogram of the fraction obtained using 0.1 N HCl followed by filtrations and ultrafiltrations. In both cases the internal standard (Phe–Phe) is indicated. The biggest peak nearby is the amino acid tryptophan.

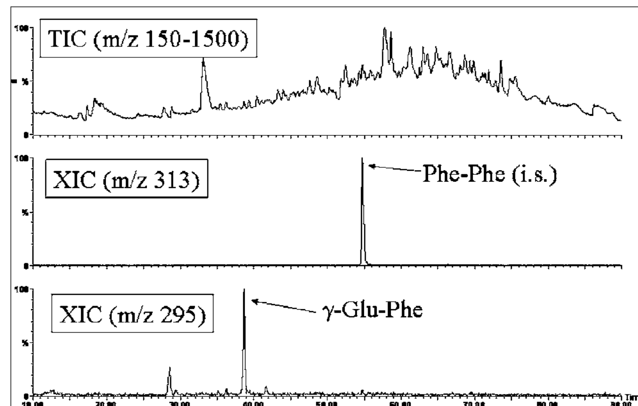

Figure 2. HPLC-ESI/MS profiles of Parma dry-cured ham oligopeptide fraction extracted from a sample that underwent standard processing: total ion chromatogram (TIC, top) obtained by monitoring all mass signals included between *m/z* 150 and 1500; extract ion chromatogram (XIC) obtained from the TIC by extracting only the signal at *m/z* 313, corresponding to the protonated molecular ion of the internal standard Phe–Phe (middle); XIC chromatogram obtained from the TIC by extracting only the signal at *m/z* 295, corresponding to the protonated molecular ion of γ-Glu–Phe (bottom).

marker, the value of which is to be complemented with the study of the peptide and amino acid fraction.

Peptide Extraction, Analysis, and Identification. The use of deproteinizing agents such as acetonitrile, ethanol, and perchloric or trichloroacetic acid is a common procedure to obtain the ham extract for peptide analysis (*7*, *17*). According to the type and concentration of the deproteinizing agent, a first selection is carried out with reference to the soluble peptide fraction: on average, these methods allow only small to medium peptides with hydrophilic properties to be recovered. Other authors developed a different extraction method based on the use of HCl as a nonprecipitating extracting agent to solubilize peptides with molecular masses up to several kilodaltons to be analyzed by HPLC (*4*) and HPLC-MS (*15*). An internal standard, L-phenylalanyl-L-phenylalanine (Phe−Phe), to allow a semiquantitation of the peptides, was added, and a number of physical fractionation steps with increasingly selective filtrations and ultrafiltrations were performed on the mixture obtained.

In the case of the oligopeptide fractions extracted from Parma hams, a comparison was made between the procedure based on selective filtrations and ultrafiltrations (see Materials and Methods) and the use of 5% trichloroacetic acid as deproteinizing agent according to a previous study (*18*). **Figure 1** shows the chromatographic profiles of the peptide fractions obtained by using the deproteinizing agent or fractionation by physical methods after solubilization in diluted HCl (followed by filtrations and ultrafiltrations at 10 and 3 kDa): major differences are detectable at high retention times, where higher molecular weight and/or more hydrophobic peptides are eluted (*15*). In the 5% trichloroacetic acid soluble fraction only peaks at retention times lower than 40 min were detected, indicating that bigger and/or hydrophobic oligopeptides were precipitated. On the basis of these results, the filtration−ultrafiltration with 10 and 3 kDa cutoff filters after solubilization in diluted HCl was performed, in order to allow for a more complete analysis of the oligopeptide fraction also including peptides longer than three or four residues.

All of the peaks of the peptides identified were integrated by using the extract ion chromatogram (XIC) technique (*6*), obtained from the original total ion chromatogram (TIC), which allowed a better integration to be achieved (**Figure 2**). The semiquantitation was obtained by calculating, for every peptide of interest, the ratio between the peptide peak area in the corresponding XIC and the peak area relative to the internal standard Phe−Phe, according to the method used in a previous study (*18*).

Although hundreds of different peptides can be identified in the different fractions extracted from the Parma hams, only representative oligopeptides were chosen for the semiquantitation and for the statistical analysis in all of the samples. In particular, the oligopeptides traced in all samples were those that were particularly abundant at least in one of the three different groups characterized by different processing times. In this way it was possible to study a group of oligopeptides that was at the same time typical of the Parma ham and possibly related to the aging time. On the whole, 34 peptides with molecular masses ranging from 216 to 8373 Da were semiquantified in all samples. The list of the selected peptides is reported in **Table 2**.

It may be noted that, although ultrafiltration with filters with molecular cutoff at 3000 Da was performed, many peptides (actually one-third of the selected) show a molecular mass higher than this value. This can be explained by considering that the value indicated for the ultrafilters is a nominal value based on an average pore size, which is matched with an average protein molecular mass, by considering every protein to be roughly spherical. A platicurtic pore size distribution around the average

Table 2. Peptides Traced in the Parma Dry-Cured Hams

oeak	molecular mass (Da)	RT ± 0.2 (min)	possible identification
1	246	26.5	NN,[a] DL(I),[a] L(I)D,[a] PM,[a] MP,[a] EV,[a] VE[a]
2	228	29.9	PL(I),[a] L(I)P[a]
3	222	31.1	GF (*m/z* 166 **y1**)[b]
4	260 (1)	33.0	γ-EI[c]
5	260 (2)	34.5	γ-EL[c]
6	294	37.5	γ-EF (*m/z* 166 **y1**)[b],[c]
7	216	39.0	VV,[a] TP,[a] PT[a]
8	319 (1)	40.5	DW (*m/z* 205 **y1**, *m/z* 188 **z1**)[b]
9	244 (1)	41.8	L(I)L(I),[a],[d] EP,[a] PE[a]
10	319 (2)	42.0	WD[a]
11	244 (2)	44.2	L(I)L(I),[a] EP,[a] PE[a]
12	422	47.0	YGAL(I), YAGL(I) (*m/z* 292 **b3**, 260 **y3**)[b]
13	934	48.0	
14	2114	49.8	
15	2458	50.4	
16	343 (1)	51.4	VL(I)L(I) (*m/z* 245 **y2**, 213 **b2**)[b]
17	3267	55.3	
18	343 (2)	55.5	L(I)VL(I) (*m/z* 231 **y2**, 213 **b2**)[b]
19	3164	56.6	
20	4969	58.8	
21	2531	60.7	
22	3080	61.6	
23	2684	64.0	swine pyruvate kinase f(1–25)[d]
24	971	65.0	
25	4373	66.5	
26	490	68.0	VL(I)L(I)F (*m/z* 392 **y3**, 326 **b3**, 279 **y2**, 213 **b2**)
27	3239	68.4	
28	6942	70.7	
29	8372	72.0	
30	3571	72.1	
31	7055	72.3	
32	8253	72.7	
33	2856	72.8	
34	8292	73.8	

[a] The reported sequence is consistent with the found molecular mass, but no diagnostic fragments have been identified in the mass spectrum. Amino acid residues in peptide sequences are given according to IUPAC-IUB Joint Commission on Biochemical Nomenclature (JCBN), Nomenclature and Symbolism for Amino Acids and Peptides, Recommendations 1983. [b] The reported sequence has been assigned on the basis of diagnostic fragments identified in the mass spectrum (indicated in parentheses). [c] The reported sequence has been assigned by comparison with an authentic sample (*19*). [d] This peptide has been identified in a previous work (*20*). Mass peaks are labeled by following the standard notation used for peptide fragments (bold character): "**b**" and "**y**" peaks indicate the ions generated by the fragmentation of the amidic bond, i.e., an N-terminal fragment ("**b**") or a C-terminal fragment ("**y**"), whereas "**z**" peaks are the C-terminal ions due to the fragmentation of the bond between the CO group and the α-carbon. The numbers after the labels indicate how many amino acid residues are included in the fragments.

value may be postulated, and the presence of peptides with a very compact structure may easily account for the higher molecular mass peptides observed in our experiments.

As can be seen in **Figure 1** and from the retention times reported in **Table 2**, the majority of the peptides elute between 20 and 75 min: in this range the peptides elute according to both their molecular mass (short peptides elute first, longer peptides elute later) and to their lipophylicity (hydrophilic peptides elute first, lipophilic peptides elute later).

All of the selected peptides were labeled on the basis of their molecular masses (easily identified in the associated mass spectra) and their chromatographic retention times, whereas very little information was available about their sequence, given the scarce fragmentation induced in the ESI source by the single-stage MS instrument utilized. In the case of hard cheeses, it had been previously demonstrated that the information obtained even from this low fragmentation can be suitably combined with the available information on the possible protein sequences which originated from the peptides to reach a complete identification of the amino acid sequence (*15*). However, it should be noted that, in the case of the hard cheeses, peptides mainly were from only four proteins, namely, α_{S1}-, α_{S2}-, β-, and κ-casein. In the case of the ham samples, this approach does not seem to be feasible, given the enormous number of possible protein sequences from which the peptides can be derived: actually, the peptides can be formed by the proteolysis of both myofibrillar and sarcoplasmatic proteins, accounting for hundreds of possible sequences. The limited number of possible amino acid combinations and/or the in-source collisionally induced dissociation (CID) allow a tentative assignment of the peptide sequences only for a few short peptides with molecular mass < 500 Da (**Table 2**). Many peptides for which it has been possible to get information about the sequence are characterized by one or more hydrophobic residues: nearly all of the short peptides identified as characteristic of Parma ham contain at least one, but often two, three, or four, hydrophobic residues, such as valine, phenylalanine, tryptophan, leucine, or isoleucine (the last two cannot be univocally distinguished in a mass spectrum, being isomers). The most striking examples are peptides corresponding to peaks 16 [Val−Leu(Ile)−Leu(Ile)], 18 [Leu(Ile)−Val−Leu(Ile)], and 26 [Val−Leu(Ile)−Leu(Ile)−Phe]. In particular, the last one, although composed of only four amino acids, elutes very late, due to its high hydrophobicity. Peaks 4, 5, and 6 have been previously identified as γ-Glu−Ile, γ-Glu−Leu, and γ-Glu−Phe, respectively, by comparison with a real specimen synthesized by solid-phase synthesis (*19*); the identification was made on the basis of the comparison of retention times of analytes and standards and confirmed by spiking the ham samples with pure standards. The sequence of the peptide corresponding to peak 23 was previously identified by Edman degradation as the N-terminal part of swine pyruvate kinase (*20*). For all other peptides only the molecular mass has been reported.

Composition of the Peptide and Amino Acid Fractions at Various Aging Times and Effects on Ham Flavor. The effect of the processing time on the pattern of the oligopeptides and free amino acids generated in Parma dry-cured ham was evaluated at the established manufacturing deadlines (**Tables 3** and **4**). The General Linear Model used for the analysis of peptides and free amino acids included three main effects, that is, the session of fresh ham selection at the slaughterhouse (four times), the dry-curing plant (two factories), and the processing time (three deadlines). Although the first two effects were included in the model as possible known sources of variation, the processing time was the lone main effect focused and reported in **Tables 3** and **4**. Fresh hams selected at the slaughterhouse at each session were homogeneously split and assigned to both manufacturing plants; then, in each curing batch, a similar number of raw hams underwent different processing times (450, 570, and 690 days). Interactions between main effects were not calculated. To take into account the influence of the pH of the raw meat (pH_{24h}) on the proteolysis of dry-cured ham (*23*), the pH was included as covariate in the General Linear Model, and the estimated coefficients are reported in **Tables 3** and **4**; when significant ($P < 0.05$), it denoted a relationship between pH_{24h} and the corresponding peptide or amino acid. The estimate of the experimental error of the model including the main effects and the covariate was calculated and reported as the pooled standard error of the mean (SEM).

Table 3. Effect of Aging Time on Oligopeptide Semiquantitative Amounts ($Area_{peptide}/Area_{internal\ standard}$) and Correlation with the Ultimate pH of the Fresh Ham (pH_{24h})[a]

molecular mass (Da)	processing time 450 days (*n* = 24)	570 days (*n* = 24)	690 days (*n* = 24)	SEM[b]	covariate (pH_{24h}) estimate coefficient[c]
<400					
216	0.160 ab	0.111 b	0.190 a	0.013	−0.14
222	0.058 b	0.072 b	0.122 a	0.006	−0.23 **
228	1.227	1.562	1.503	0.073	−3.11 **
244 (1)	0.121	0.138	0.123	0.004	−0.18 **
244 (2)	0.055 b	0.073 b	0.097 a	0.004	−0.08 *
246	0.378 ab	0.358 b	0.578 a	0.036	−1.33 **
260 (1)	0.365 c	0.518 b	0.638 a	0.014	−0.66 **
260 (2)	0.586 c	0.896 b	1.135 a	0.027	−0.93 **
294	0.142 c	0.215 b	0.294 a	0.008	−0.29 **
319 (1)	0.123 b	0.136 ab	0.179 a	0.008	−0.31 **
319 (2)	0.066 b	0.077 a	0.069 ab	0.000	−0.01
343 (1)	0.105	0.107	0.138	0.013	−0.21 *
343 (2)	0.059 b	0.105 a	0.099 ab	0.008	−0.11
<1000					
422	0.212	0.211	0.273	0.018	−0.36 *
490	0.275 b	0.522 a	0.462 a	0.030	−0.54 *
934	0.069	0.113	0.101	0.010	−0.15 *
971	0.134	0.143	0.120	0.009	−0.01
<4000					
2114	0.107	0.102	0.094	0.008	−0.31 **
2458	0.067	0.071	0.052	0.006	−0.08
2531	0.225 b	0.431 a	0.442 a	0.028	−0.36
2684	0.132 b	0.265 a	0.170 b	0.016	−0.23
2856	0.106 b	0.165 a	0.133 ab	0.010	0.31
3080	0.092 b	0.147 a	0.158 a	0.009	−0.12
3164	0.485 b	0.766 a	0.722 ab	0.046	−0.23
3239	0.286	0.001	0.002	0.017	−0.08
3267	0.048 a	0.000 b	0.001 b	0.000	−0.04 *
3571	0.039 b	0.066 a	0.024 b	0.004	−0.04
<10000					
4373	0.077 b	0.198 a	0.141 a	0.010	−0.12
4969	0.060 b	0.143 a	0.144 a	0.009	−0.15 *
6942	0.107	0.177	0.116	0.021	−0.22
7055	0.041 b	0.106 a	0.075 ab	0.009	−0.16 *
8253	0.038 b	0.093 a	0.026 b	0.008	−0.10
8292	0.037	0.053	0.044	0.004	0.09 **
8372	0.014 c	0.040 b	0.074 a	0.004	−0.05

[a] Estimated means within a row with different lower case letters are different ($P < 0.05$). [b] Values for SEM represent the pooled standard error of the mean. [c] The pH of fresh hams (pH_{24h}) was included in the model as covariate. Significant effect: *, $P < 0.05$; **, $P < 0.01$.

Table 4. Effect of Aging Time on Free Amino Acid Amounts (Amino Acid Content Expressed as Milligrams per 100 g of Protein)[a]

amino acid	taste	processing time 450 days (*n* = 48)	570 days (*n* = 56)	690 days (*n* = 54)	SEM[b]	covariate (pH_{24h}) estimate coefficient[c]
Asp	MSG-like	871 b	944 a,b	1025 a	16.6	93.1
Glu	MSG-like	1833 c	2053 b	2197 a	11.9	−463 **
Asn	sour	218	196	214	4.15	−300 **
Ser	sweet	834	806	836	10.5	−277 **
Gln	sweet	32.6 a	2.58 b	0.10 b	0.61	−6.62
His[d]	sour	742 c	875 a	812 b	8.36	−283 **
Gly	sweet	770 c	839 b	896 a	6.70	−245 **
Thr	sweet	786 b	826 b	887 a	7.39	−312 **
Arg	bitter	887	842	839	21.8	−181
Ala	sweet	1562 b	1807 ab	1857 a	17.9	−697 **
Tyr	tasteless	516	543	539	5.55	−112 **
Val	bitter	1789 c	2095 b	2225 a	14.4	−639 **
Met	bitter	488 c	548 b	666 a	5.65	−296 **
Trp	bitter	138 c	165 b	193 a	2.19	−58.7 **
Phe	bitter	735 c	836 b	883 a	7.82	−414 **
Ile	bitter	865 c	1036 b	1219 a	10.9	−504 **
Pro	sweet	799 b	956 a	1009 a	17.7	−195
Leu	bitter	1225 c	1426 b	1557 a	12.4	−590 **
Orn	unknown	115 b	182 a	213 a	6.74	−38.9
Lys	sweet	2578 b	2912 a	2979 a	28.3	−515 *
total		17694 c	19343 b	20383 a	133	−5662 **

[a] Estimated means within a row with different lower case letters are different ($P < 0.05$). [b] Values for SEM represent the pooled standard error of the mean. [c] The pH of fresh hams (pH_{24h}) was included in the model as covariate. Significant effect: *, $P < 0.05$; **, $P < 0.01$. [d] Taste of His·HCl (*27*).

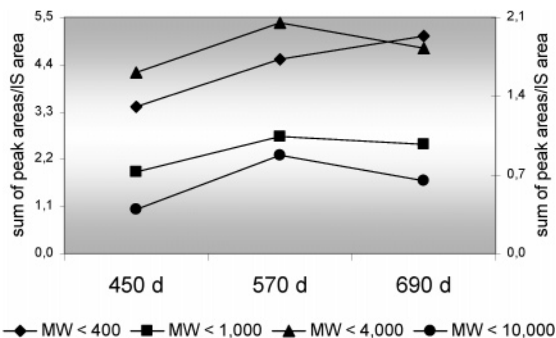

Figure 3. Changes in Parma dry-cured ham peptide fractions with reference to the length of processing time and molecular weight (MW) range. Semiquantified peptides are grouped according to MW (**Table 3**), summed within each MW range, and displayed at each assayed aging step. Each point was computed as the sum of the ratio of selected peak areas to that of the internal standard (α-Phe–Phe) peak area. Only the sum of peptides with molecular mass < 400 Da was plotted with reference to the left *Y*-axis.

Peptides may be regarded as intermediate molecules, which are generated during the proteolysis and are broken down by further proteolytic activity. Because the activity of the proteolytic enzymes is expected to be characteristic of the process and it changes during the aging time, the peptide pattern is strictly dependent upon these factors. Thus, a detailed study of the peptide fraction should allow long-aged hams to be distinguished from less aged hams, although other indices such as free amino acids and bulk proteolysis values were similar (*11*).

Most small peptides below 400 Da (di- and tripeptides) increased with increasing processing time, as a result of the more protracted exposure to the action of still active proteolytic enzymes (*21*) and the availability of large peptides formed during aging to be used as substrates. Because small peptides are formed toward the end of the proteolytic process, which goes from intact proteins to free amino acids, it is reasonable to expect a more effective generation rate in the case of protracted aging deadlines (*8*, *17*).

If the trend of short peptides is to increase during the aging time, in contrast, the high molecular mass peptides, which are closer to the intact proteins, show a tendency to accumulate in the first part of the aging period and to decrease in the last part, consistent with their double role of products and substrates of the proteolytic activity. The sums of semiquantified oligopeptides falling into each molecular mass range given in **Table 3** were computed and are displayed in **Figure 3**, showing that most peptides over 400 Da decreased during the period between 570 and 690 days. In this period of time, the dynamic state between generation and degradation has definitely shifted toward the degradation of the peptides previously generated. Thus, extended processing (570 days) was characterized by the highest content of medium-high molecular mass peptides, whereas the extended aging (690 days) was characterized by a sharp decrease of high molecular mass peptides and, accordingly, by an increase of di- and tripeptides. Some high molecular mass peptides show a decreasing trend starting from the 450 day processing period

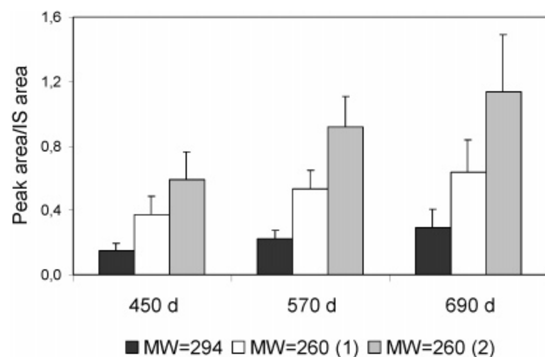

Figure 4. Increase of γ-glutamyl dipeptides detected in Parma dry-cured hams with reference to processing length. Peaks with molecular weights of 260 (1), 260 (2), and 294 were identified as γ-Glu–Ile, γ-Glu–Leu, and γ-Glu–Phe, respectively. The amount was computed as the ratio of the γ-glutamyl dipeptide peak area to that of the internal standard (α-Phe–Phe) peak area. Vertical thin bars represent standard deviation.

(**Table 3**). Among them, it is worth mentioning that the peptide with a molecular mass of 3267 (peak 17) can be considered as a marker of the traditional processing, because it is completely absent in the samples of 570 and 690 days.

The γ-glutamyl dipeptides are to be considered separately, given their particular molecular structure including a γ-glutamyl amidic bond. These peptides undergo a sharp increase at extended aging times, as evident in **Table 3** and **Figure 4**. Whereas α-peptides may be broken down by proteolytic enzymes to free amino acids, γ-glutamyl dipeptides increase with aging time, being less affected by peptidases. Accordingly, the generation of γ-glutamyl dipeptides may have a double link with proteolysis and protracted aging times. These dipeptides cannot be regarded as simple proteolytic products, but can be formed by an in situ reaction between free amino acids released from muscle proteins catalyzed by γ-glutamyltranspeptidases (*22*). According to this hypothesis, the donor substrates of the glutamyl group can preferentially be glutamine (which shows a sharp decrease in the amino acid pool), glutathione, and glutamic acid. The high number of variables possibly involved in the formation of γ-glutamyl dipeptides (available glutamyl substrates, activity of γ-glutamyltranspeptidase, and free amino acids with particular reference to hydrophobic amino acids) accounts for the variability shown by the amounts of these peptides in hams aged for a longer period (690 days) (**Figure 4**). According to the postulated mechanism, these dipeptides are strongly linked to the proteolysis degree and the aging time and may be effective markers of long-aged dry-cured meat products.

Data reported in **Table 3** confirm the pH effect upon most small peptide amounts ($P < 0.05$), with an increase in most peptides in the case of low pH, as indicated by the negative estimate coefficient of covariate. Actually, at low pH of the muscle, enhanced release of cathepsins from lysosomes (*24*) may account for the more abundant generation of oligopeptides. Accordingly, in the case of dark firm dry (DFD) pork meat, a condition notoriously linked to high pH, a lower amount of polypeptides at 2 h post-mortem as compared to other types of pork meat has been found (*25*). In the case of the dry-cured hams here assayed, the small peptides below 400 Da, which are formed in the final steps of the proteolytic pathway, were found to be more significantly affected by the acidic pH than longer oligopeptides (in the range of 1000−10000 Da).

The effect of the peptides on ham flavor is very well-known: several small peptides have been reported to be bitter, and their increase with aging is reported by several authors (*4*, *5*, *7*). A sharp rise of small peptides as compared to free amino acids has been reported in very aged hams (*5*, *16*). Nevertheless, on average, sensory evaluation of flavor-related traits of dry-cured ham improves by increasing the ham processing time (*11*, *26*). In this respect, the role of bitter peptides may be counteracted by the combination with other taste-active compounds (amino acids, salt, other peptides) in adequate proportion, whereas an unbalanced accumulation could generate an unpleasant bitter taste. In our case, small peptides bearing hydrophobic residues are formed, some of them already found to be related to the bitter taste in aged hams, such as Gly−Phe and Leu−Leu (*6*). However, there are also molecules eventually able to counteract this undesired taste, for instance, the γ-glutamyl dipeptides: the enzymatic synthesis of γ-glutamyl dipeptides has been described as a tool for masking the bitterness of some amino acids (*22*). If compared to the taste of single amino acid residues (glutamic acid = monosodium glutamate-like; leucine, isoleucine, phenylalanine = bitter), glutamyl dipeptides are by far less bitter, although taste intensities of peptides are generally weaker if compared to those of the corresponding amino acids (*27*). Therefore, the pleasant ham flavor may ultimately emerge from the correct balancing of different enzymatic activities, such as the proteolytic production of hydrophobic amino acids and small peptides and the generation of γ-glutamyl dipeptides masking their unpleasant bitterness.

Further information will be gained from the identification of the peptide sequences: in this case it might be possible to predict whether a particular peptide will give a bitter taste, in agreement with the number and the hydrophobic values of the amino acid residues.

Free Amino Acids. The amount of free amino acids in Parma dry-cured ham was closely related to the salt content and to the overall proteolysis (*14*). The possible changes of the free amino acid amount and pattern due to different extended processing times are reported in **Table 4**.

An overall decrease of free amino acids with very extended aging (600 days) was reported (*5*) in the case of Iberian ham and ascribed to the degradation to volatile compounds (*16*). In the case of Parma hams, most amino acids increased during extended aging, whereas a few amino acids maintained a similar value (asparagine, serine, arginine, tyrosine) or decreased (glutamine and histidine). For asparagine and glutamine the deamidation to ammonia and aspartic and glutamic acid, respectively, may be postulated; glutamine may even be the substrate for the production of γ-glutamyl peptides (*19*). In the case of tyrosine the increase in ham muscle may be masked by the low solubility and/or precipitation as white film or chalks (*28*). Arginine, tyrosine, and histidine may be suitable substrates for decarboxylation to biogenic amines (*29*), due to the possible growth of microorganisms with decarboxylase activity inside hams (*30*). The content in branched chain amino acids valine, leucine, and isoleucine, which are known to be primarily involved in the generation of branched volatile compounds and identified in the headspace of longer-aged ham (*2*), in the current samples was found to be enhanced by prolonged aging (**Table 4**). The BCAA amount proved to be highly and positively related to the proteolysis degree of dry-cured ham (*31*), and the rise of the proteolysis index (**Table 1**) due to the extension of the processing time accounts for the progressive increase in BCAA, even though they partially suffer from degradation to volatile compounds.

The effect of low pH in increasing proteolysis and releasing low weight nitrogen molecules was confirmed also for the amino

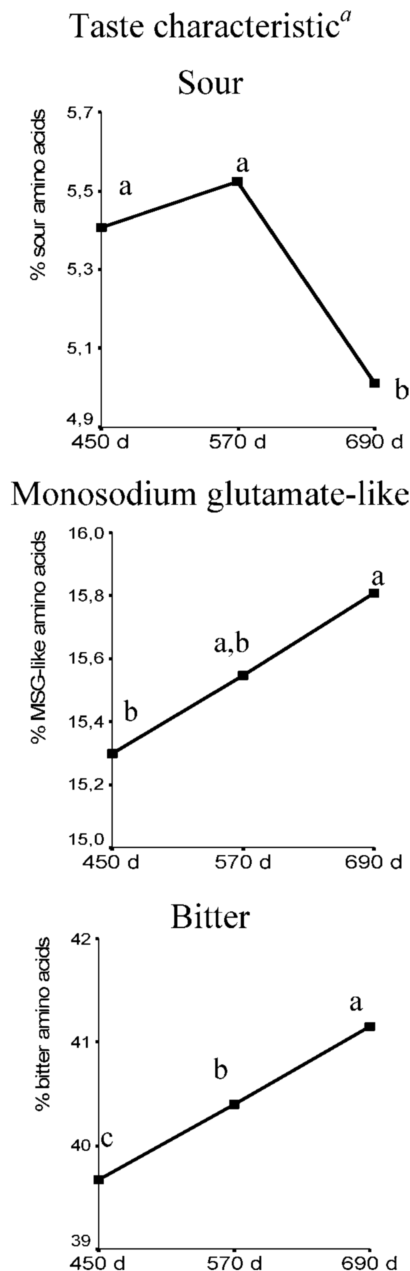

Figure 5. Effect of processing length on free amino acids taste categories in Parma dry-cured ham. [a]Sour, His + Asn; MSG-like, Asp + Glu; sweet, Ala + Gly + Ser + Thr + Lys + Pro + Gln; bitter, Arg + Ile + Leu + Met + Phe + Trp + Val, according to ref *27*. Different letters at displayed processing times indicate significant differences ($P < 0.05$, Bonferroni *t* test). Sweet-taste amino acids are not shown because the effect of processing length was not significant.

acids (**Table 4**). Although proteolytic enzymes directly involved in amino acid release are partly inhibited by low pH, other endopeptidases releasing peptides suitable to be cleaved by exopeptidases are activated by low muscle pH (*21*, *32*), thus favoring further release of amino acids. Degradation processes postulated for some amino acids during dry-cured ham processing (glutamine, arginine, etc.) could account for the lack of pH influence over their content; the ornithine increase, ascribed to arginine catabolism and unrelated to proteolytic events (*33*), is independent of muscle pH.

Protracted processing times yielded an increase of hydrophilic, umami-enhancing amino acids (aspartic and glutamic acid) and in hydrophobic, bitterness-enhancing amino acids (*27*, *34*). Thus, the increase in aspartic and glutamic acids with aging time is consistent with previous results showing a positive correlation with cured/aged taste perception (*2*, *8*). The increase of hydrophobic amino acids with aging was not accompanied by bitterness enhancement in aged Parma ham (*11*), a finding in contrast with that of other authors (*5*) who found a tendency for bitterness to increase in the case of traditional processed Iberian hams when compared to shorter processing.

Taste properties of dry-cured hams may be more accurately described by free amino acids grouped on the basis of their common taste characteristics rather than by single amino acids (*2*, *17*). Free amino acids were grouped and combined in agreement with taste categories [sweet, bitter, sour, monosodium glutamate (MSG)-like], and the relative quantities of amino acids with common taste properties were calculated with respect to the total free amino acids. Changes of amino acid taste categories as a result of different processing times are reported in **Figure 5**, showing that taste classes "sour", "MSG-like", and "bitter" were significantly influenced by processing times. Because the sensory evaluation of dry-cured ham has been reported to improve according to prolonged ham aging (*11*, *26*), it seemed to be reasonable to postulate that a moderate percentage increase of bitter amino acids is balanced by the increase in MSG-like amino acids and salt (**Table 1**) and by the decrease of sour amino acids and moisture, thus positively influencing the flavor.

The pH of the fresh ham affected ($P < 0.001$) both the amount and the pattern of free amino acids: in hams characterized by low pH_{24h}, bitter and sour amino acids are released in higher amount (estimated pH coefficients of −3.36 and −1.29 for bitter and sour taste, respectively), whereas umami-tasting amino acids are less abundant (estimated pH coefficient = 2.64). Accordingly, the ultimate pH of the fresh ham influences the taste of very aged dry-cured hams.

Conclusion. Because the taste of dry-cured ham is largely attributable to nonvolatile molecules such as oligopeptides and free amino acids, it can be assumed that it is also influenced by the manufacturing length. The amount and the pattern of these nonvolatile components of Parma ham are related to fresh ham quality (ultimate pH) and to the extent of processing time. The amount of most peptides identified and semiquantified with a molecular mass in the range of 200−1000 Da increased up to 690 days of processing (extended aging) and showed a negative relationship with the ultimate pH of fresh ham. As a consequence, it seems that, even in case of extended aging, muscle properties (salt, moisture, enzyme activities, pH) favor the generation rather than the hydrolysis of these peptides.

Peptide mapping, although representing a dynamic state, may provide useful information for characterizing a selected aging time: some oligopeptides in the range of 4000−10000 Da are more abundant in extended-processing dry-cured hams (570 days), whereas small peptides and, above all, the γ-glutamyl dipeptides, may be regarded as markers of very aged dry-cured hams. Both extended aging and low ultimate pH of fresh hams yielded an increase of most free amino acids, more remarkable for bitter amino acids. The MSG-like components, in terms of aspartic and glutamic acids and γ-glutamyl dipeptides, seem likely to account for the improvement of the sensory properties of long-aged hams. These results can be helpful for improving the actual knowledge about the combination of taste-active compounds (free amino acids, peptides, salt, and moisture) for enhancing the perception of the aged taste.

ABBREVIATIONS USED

PDO, protected designation of origin; BCAA, branched chain amino acid; XIC, extract ion chromatogram; TIC, total ion chromatogram.

ACKNOWLEDGMENT

Cinzia Grisendi, Paola Lasagni, Francesca Bellengi, Lorenza Corradi, and Francesca Beretti are gratefully acknowledged for their collaboration.

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Received for review May 9, 2006. Revised manuscript received September 14, 2006. Accepted September 22, 2006. The work presented in this paper was supported by a funding contribution by the Emilia-Romagna Region (Italy) to the project "Valorizzazione nutrizionale ed organolettica del Prosciutto di Parma".

JF061312+