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Protein Carbonylation and Water-Holding Capacity of Pork Subjected to Frozen Storage: Effect of Muscle Type, Premincing, and Packaging

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ABSTRACT: The present work aimed to study the relationship between protein carbonylation and the loss of water-holding capacity (WHC) occurring during frozen storage of porcine muscles. Pork samples corresponding to two different muscle types, glycolytic M. longissimus dorsi (LD) and oxidative M. psoas major (PM), and subjected to two different premincing, minced (MINCED) and intact pork pieces (INTACT), and packaging, vacuum packaged (VACUUM) and packed in oxygen-permeable bags (OXYGEN), procedures were frozen (−18 °C/12 weeks) and analyzed at sampling times upon thawing (weeks 1, 4, 8, and 12) for the relative amount of specific protein carbonyls, α-aminoadipic and γ-glutamic semialdehydes (AAS and GGS, respectively), and their ability to hold water using two different techniques. The formation of protein carbonyls occurred concomitantly with the loss of WHC, and both phenomena were found to be more intense in LD muscles and in MINCED and OXYGEN pork samples. The loss in WHC was from ca. 10 to 30% in 12 weeks, depending on the method of determination. Plausible mechanisms by which protein carbonylation may decrease the WHC of pork samples are thoroughly discussed in the present paper. Besides the likely impact of protein carbonylation in the water−myofibrillar protein relationships, the implication of AAS and GGS in further reactions including plausible cross-linking would explain the decrease of these semialdehydes by the end of frozen storage and would reinforce their liability in the loss of WHC of porcine muscles. The exact nature of these reactions, however, should be investigated in further studies.

KEYWORDS: frozen storage, pork, protein oxidation, water-holding capacity, α-aminoadipic semialdehyde, γ-glutamic semialdehyde

INTRODUCTION

Freezing is the most frequently used technology to preserve fresh meat during long-term storage. Keeping meat under frozen storage enables the meat industry to (i) adapt its offerings to consumers' demand, (ii) adjust the meat supply to the processing rate, and (iii) transport meat to distant importing countries. Microbial spoilage fully stops in frozen meat while certain (bio)chemical reactions are still active and will lead to undesirable quality changes.[1,2] The onset of lipid oxidation during frozen storage of meat has been profusely studied, and it leads to deleterious effects on the color, texture, and flavor of muscle foods.[3−5] However, the major threat to the quality of frozen meat is the decrease of water-holding capacity (WHC), which is manifested as a loss of exudate (drip) upon thawing as well as lower water-holding in products. The fluid purge has a direct impact on the weight of meat and reduces its tenderness and the overall eating quality, with all of these factors seriously affecting its commercial value.[3] The volume of drip produced has been directly linked to the rate of freezing, which in turn has been related to the size and location of ice crystals in frozen meat.[2,3] The intrinsic mechanism is generally ascribed to the combination of multiple factors including the physical damage caused by ice crystals on sarcolemma and the structure of myofibrils and the chemical damage caused by solute concentration, which eventually leads to water migration and fluid loss.[2]

Muscle proteins are also affected by freezing temperatures, and until recently, the only processes reported to take place were their denaturation and their proteolytic degradation.[2,5−7] However, some recent studies have demonstrated that meat proteins also undergo oxidative reactions to yield protein carbonyls during frozen storage of pork and poultry.[8,9] Carbonylation is one the most remarkable changes in oxidized muscle proteins and occurs as a result of the oxidative degradation of particular amino acids such as lysine, arginine, proline, and threonine.[10] In fact, the estimation of the total amount of protein carbonyls using the dinitrophenylhydrazine (DNPH) method has been profusely employed as an indicator of protein oxidation (Pox) in multiple meat products.[11−14] In other cases, protein oxidation is manifested as a loss of functional groups and modification of side chains, loss of tryptophan fluorescence, peptide scission, and formation of intra- and intermolecular cross-links.[15−17] The influence of Pox on meat quality is still poorly understood, but it is generally accepted that it leads to undesirable texture changes in raw meat and processed muscle foods and a loss of nutritional value (reviewed by Lund et al.[18]). Myofibrils make up between 82 and 87% of the muscle cell volume, and around 85% of water is mostly physically entrapped inside this protein structure.[19,20] Hence, the severe modifications induced by Pox on myofibrillar proteins may have an impact on WHC and other functional properties of muscle tissue.[15,18] Recent attempts to shed light on this issue have highlighted a likely suppressing effect of Pox on

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WHC, whereas the precise chemical mechanisms involved remain unknown.[21,22] The occurrence of Pox during frozen storage of meat has never been studied in relation to its likely impact on WHC. The recent report on highly specific methodologies (liquid chromatography−mass spectrometry, LC-MS) to analyze specific protein carbonyls, namely, α-aminoadipic and γ-glutamic semialdehydes (AAS and GGS, respectively), in myofibrillar proteins[23,24] enables the accomplishment of more challenging studies to elucidate the role of protein carbonylation on the loss of WHC in meat subjected to frozen storage. AAS is formed as a result of the oxidation of lysine, whereas GGS derives from proline and arginine. Both compounds are considered to be major protein carbonyls and account for up to 60% of the total amount of carbonyls in animal proteins.[18,25] The present work was conceived to study the relationship between Pox and WHC in frozen pork through the analysis of specific protein carbonyls, AAS and GGS. The effects of the metabolic type of the muscle (oxidative vs glycolytic) and the application of different premincing and packaging technologies to meat subjected to frozen storage, on the progress of Pox and WHC, were also assessed.

MATERIALS AND METHODS

Chemicals and Meat Supply. All chemicals were supplied by Fluka Chemie AG (Buchs, Swizweland), Sigma-Aldrich (Steinheim, Germany), and J. T. Baker (Deventer, The Netherlands). Ethanol and ethyl acetate of atomic absorption spectrophotometry grade were from Rathburn Chemicals Ltd. (Walkerburn, Scotland). Distilled water used was of Milli-Q grade (Millipore Corp., Bedford, MA). Porcine muscles, M. longissimus dorsi (LD) (n = 4) and M. psoas major (PM) (n = 6), were randomly obtained from a homogeneous batch of pigs (age at slaughter = 165 days, carcass weight = 83 kg) in a local slaughterhouse in Forssa (Finland). Muscles were immediately transferred to the laboratory, analyzed for final pH (24 h) (LD = 5.34; PM = 5.48), and kept in cold storage (4 °C, overnight) until processed for the freezing experiments as explained below.

Treatments and Frozen Storage of Meat. *Effect of Freezing/Thawing.* To study the effect of freezing/thawing on Pox and WHC of pork, the day after slaughter intact meat samples (∼200 g) from both porcine muscles (LD and PS) were freed from visible fat and frozen in oxygen-permeable plastic bags (WalkiPack HD-PE, 20 μm, oxygen permeability = 2000 $cm^3/(m^2 \cdot 24\ h)$ at −18 °C for 24 h, thawed by keeping the samples in a refrigerator (4 °C) overnight, and processed for physicochemical analyses. Unfrozen counterparts from the same muscles were analyzed after 2 days of storage at 4 °C and employed as control samples.

Effect of Frozen Storage. To study the effect of frozen storage on Pox and WHC of pork, the following experimental design was carried out. The day after slaughter, the muscles were freed from visible fat and grouped into different batches depending on the metabolic profile of the muscle (glycolytic LD vs oxidative PM), a premincing treatment (MINCED vs INTACT), and the packaging employed for frozen storage (OXYGEN vs VACUUM). MINCED meat was obtained by grinding muscles to ∼3 mm particle size using a cutter in a pilot plant (Seydelmann Rasant K-40, Fa Seydelmann, Stuttgart, Germany), whereas INTACT meat refers to nonminced (intact) muscle portions. OXYGEN packaging refers to meat packaged in oxygen-permeable plastic bags (WalkiPack HD-PE, 20 μm, oxygen permeability = 2000 $cm^3/(m^2 \cdot 24\ h)$ in contrast to VACUUM-packaged meat (Knöckel Pentafilm 208, 80 μm, oxygen permeability = 53 $cm^3/(m^2 \cdot 24\ h)$. According to the factorial design applied, 8 different groups of samples (2^3) were considered and replicated 16 times, which results in 4 experimental units (n = 4) per group and per sampling time with each experimental unit accounting for approximately 200 g of meat. All samples were kept under freezing conditions (−18 °C) in the darkness during 12 weeks. At sampling times (1, 4, 8, and 12 weeks), two experimental units per batch were taken from the freezer, thawed by keeping the samples in a refrigerator (4 °C) overnight, and processed for physicochemical analyses.

Analysis of Protein Carbonyls by LC-ESI-MS. Standard AAS and GGS were synthesized in vitro from Nα-acetyl-L-lysine and Nα-acetyl-L-ornithine using lysyl oxidase activity from egg shell membrane following the procedure described by Akagawa et al.[25] AAS and GGS were analyzed in meat products following a derivatization procedure and a LC-ESI-MS technique described by Akagawa et al.[25] and Estévez et al.,[23] respectively. Meat samples (1 g) were homogenized 1:10 (w/v) in 10 mM phosphate buffer containing 0.6 M NaCl using an Ultraturrax (24000 rpm; Janke & Kunkel, Staufen, Germany) homogenizer for 30 s. INTACT meat samples were minced right before the homogenization procedure. Aliquots of 200 μL were dispensed in Eppendorf tubes and precipitated with 2 mL of 10% trichloroacetic acid (TCA) and centrifuged at 2000 rpm for 30 min. The supernatants were removed, and the resulting pellets were treated with 2 mL of 5% TCA and subsequently centrifuged at 5000 rpm for 5 min. Then, protein carbonyl groups were derivatized as follows: first, 500 μL of 250 mM 2-(*N*-morpholino)ethanesulfonic acid (MES) buffer containing 1% sodium dodecyl sulfate (SDS) and 1 mM diethylenetriaminepentaacetic acid (DTPA) were added to each sample. In addition, 500 μL of 250 mM MES buffer containing 50 mM *p*-aminobenzoic acid (ABA) and 250 mM MES buffer containing 100 mM $NaCNBH_3$ were also added. The mixture was incubated in a bath at 37 °C for 1 h and stirred regularly. Afterward, the protein was again precipitated by the addition of 500 μL of 50% TCA and centrifuged for 10 min at 10000 rpm. The supernatants were removed and the pellets precipitated with 1 mL of 10% TCA and then washed twice with 1 mL of ethanol/diethyl ether 1:1 (v/v), shaken, and centrifuged for 5 min at 10000 rpm. Then, the precipitates were hydrolyzed with 6 N HCl at 110 °C for 18 h. The protein hydrolysates were dried using a rotatory evaporator at 40 °C, and finally the dried extracts were redissolved in 200 μL of Milli-Q water. Samples (2 μL) were injected into an Agilent 1100 series HPLC (Agilent Technologies, Palo Alto, CA) equipped with a Luna reversed-phase (RP) column (5 μm C_{18} II column, 150 × 1.00 mm i.d., Phenomenex Torrance, CA) eluted at a flow rate of 50 μL/min with isocratic water−2.5% acetic acid (solvent A; 95%) and methanol−2.5% acetic acid (solvent B; 5%). The column was operated at a constant temperature of 30 °C. MS analysis was carried out on an Esquire-LC quadrupole ion trap mass spectrometer equipped with an ESI interface (Bruker Daltonics, Bremen, Germany) and LC-MSD Trap software, version 5.2 (Bruker Daltonics). Capillary voltage was 3500 V; capillary exit offset, 25 V; skimmer potential, 15 V; and trap drive value, 36. Conventional ESI-MS data were recorded using a scan range of m/z 100−700. Nebulizer (nitrogen) pressure was 50 psi; dry gas (nitrogen) flow, 8 L/min; and dry temperature, 300 °C. Identification of both semialdehydes was confirmed by positive matches for retention time, mass spectra, and fragmentation pattern with the standard compounds. The peaks corresponding to the molecular ions of AAS-ABA and GGS-ABA were manually integrated from extracted ion chromatograms (EIC) and the resulting areas used as arbitrary indicators of the abundance of both semialdehydes (arbitrary area units, AAU). These values were corrected according to the protein content of each meat sample by multiplying by a correction factor ($C_f = P_t/P_s$), where P_t is the total average of the protein contents from all meat samples and P_s is the mean of the protein content from each meat sample. Protein was calculated on the meat samples according to the Kjeldhal method as described by Armenteros et al.[24] The whole procedure was carried out in duplicate for each meat sample.

Analysis of WHC of Meat. The WHC was assessed in completely thawed pork samples following the laboratory sausage method described

Table 1. AAS and GGS in Fresh (Unfrozen) and Frozen/Thawed Pork Samples

	unfrozen pork			frozen/thawed pork		
	LD	PM	p value[a]	LD	PM	p value[a]
AAS	2.32 ± 0.54	4.33 ± 0.70	*	4.17 ± 0.84	5.66 ± 0.74	ns
GGS	1.16 ± 0.31	4.03 ± 0.63	*	5.41 ± 0.91	6.13 ± 0.80	ns

[a] *, $p < 0.05$; ns, nonsignificant.

by Puolanne and Ruusunen[26] and the centrifugation method reported by Hamm and Grau.[27] Both procedures were carried out in duplicate for each meat sample.

The first method involves a miniature scale sausage preparation and is based on the addition of excess water to the sausage batter, resulting in the separation of water on the surface under the casing during cooking and cooling. Sausage batters were prepared with 60 g of pork from experimental batches, 40 g of pork back-fat, 140 g of distilled ice−water (to keep the temperature below 20 °C during the chopping), 4.8 g of sodium chloride, and 1.03 g of sodium polyphosphate. The ingredients were mixed and thoroughly chopped in a kitchen chopper (Moulinex Moulinette, manufactured by Moulinex, France), stuffed into a 45 mm collagen casing (manufactured by Naturin-Werke, Weinheim, Germany), cooked in a hot water bath at 75 °C to achieve an end point product temperature of 72 °C (30 min), and cooled in an ice−water bath for 30 min. The sausages were stored in a cooler at 3 °C overnight. The water released after cooking and cooling was manually separated from the sausage after peeling, and the remaining sausage was weighed. The WHC was calculated on the basis of the amount of water remaining in the cooked cooled sausage. The WHC was expressed as grams of held added water in 100 g of meat.[28] In addition, the cooking yield was calculated and expressed in percentage. The drip loss formed during thawing was mixed back into the meat during the preparation of the sausage batters and counted in the weight of meat.

The centrifugation method involved the preparation of a homogenized mixture with 20 g of pork from experimental batches, 20 g of distilled water, and 20 g of sodium chloride. The mixture was then cooked by immersion in a hot water bath at 75 °C for 25 min. Upon cooling at room temperature, the mixture was centrifuged at 3000 rpm for 10 min, and the purged water was measured. The results were expressed as percentage of added water held.

Statistical Analysis. Four experimental units per batch were analyzed in duplicate for all physicochemical analyses ($n = 8$). Raw data obtained from the analyses were computed using a three-way analysis of variance (ANOVA)[29] to study the effects of muscle type, premincing, and packaging for the amount of protein carbonyls and the WHC of meat together with the interactions. In addition, paired comparisons between means from different muscle types (LD vs PM) and from samples subjected to different mincing (MINCED vs INTACT) and packaging (OXYGEN vs VACUUM) technologies at a particular freezing time (1, 4, 8, and 12 weeks) were carried out by using Tukey's tests. Pearson's correlation coefficients were also calculated to establish relationships between protein carbonylation measurements and the WHC of pork samples.

RESULTS AND DISCUSSION

Effect of Freezing/Thawing on Protein Carbonylation. Raw pork samples were analyzed for the impact of freezing (24 h) and subsequent thawing on the formation of protein carbonyls. As expected, raw (unfrozen) muscles had small amounts of both semialdehydes. AAS and GGS are common products from oxidative stress in living tissues such as liver, plasma, and muscle,[30,31] and, hence, their presence in small quantities would reflect the in vivo formation of both compounds in porcine muscles. Significant differences ($p < 0.05$) were found between the two muscles analyzed, LD and PM, as the former had lower amounts of AAS and GGS than the latter (Table 1). Whereas it has been the subject of considerable debate, it is generally accepted that muscles rich in oxidative fibers, such as PM, are more prone to lipid oxidation than muscles rich in glycolytic ones.[32,33] This effect is usually attributed to the larger amount of phospholipids and myoglobin in oxidative muscles than in the glycolytic counterparts.[34] A more intense oxidative degradation of lipids in oxidative muscles may lead to a larger oxidative stress in proteins from such muscles because oxidative reactions between lipids and proteins are believed to be timely connected.[18] Furthermore, myoglobin and nonheme iron are directly implicated in the formation of AAS and GGS,[35] and oxidative muscles contain larger amounts of such oxidation promoters than glycolytic muscles. After the short freezing−thawing cycle, the amounts of AAS and GGS in LD were similar ($p > 0.05$) to the amounts assessed in PM (Table 1). These results suggest that the impact of freezing−thawing was more intense in LD than in PM.

Effect of Freezing/Thawing on WHC. The impact of freezing (24 h) and subsequent thawing on WHC of pork was negligible. There was drip formed upon thawing, but the drip was added back to the meat before accomplishing the WHC analyses as already explained under Materials and Methods. Taking this into the account, the capacity of frozen/thawed pork samples to hold water (137.88 g of held water/100 g of meat) was similar ($p > 0.05$) to that displayed by unfrozen samples (138.87 g of held water/100 g of meat). The behaviors of LD and PM were similar in this sense. These results suggest that the mechanisms involved in the loss of drip from meat upon thawing might not predict the capacity of the muscle tissue to bind water during subsequent processing, as assessed in the present study. The occurrence of osmotic changes during water freezing at −18 °C leads to the mobilization of water from sarcoplasma to the intercellular space. The mobilized water is only partially reabsorbed into myofibrils during thawing as the physicochemical damage caused by ice crystals in myofibrils and the sarcolema hinders the total rehydration, hence causing the eventual drip loss.[2] In the present study, the thawed pork samples were able to bind the purged water plus some extra water added during the experiments devoted to the assessment of the WHC of meat. Apparently, the significant role played by the myofibrillar and sarcolemmal structures on the purge events observed after a freezing−thawing cycle might not be influential when the technological properties of muscle tissue, such as the WHC, are tested during the manufacture of chopped meat batters. In fact, the loss of the WHC of meat is usually ascribed to changes affecting muscle proteins and occurring during long-term freeze storage.[2]

Effect of Freeze Storage on Protein Carbonylation. The progress of the relative amounts of AAS and GGS during the frozen storage of pork was analyzed in two different porcine muscles (LD vs PM) (Figure 1) and in pork samples subjected to different mincing (MINCED vs INTACT) (Figure 2) and packaging (OXYGEN vs VACUUM) (Figure 3) procedures. As a common pattern between Figures 1−3, the areas corresponding to both semialdehydes significantly increased during the first stages of frozen storage at −18 °C. The present results confirm that muscle proteins undergo oxidative reactions during frozen storage of meat, which leads to the formation of AAS and

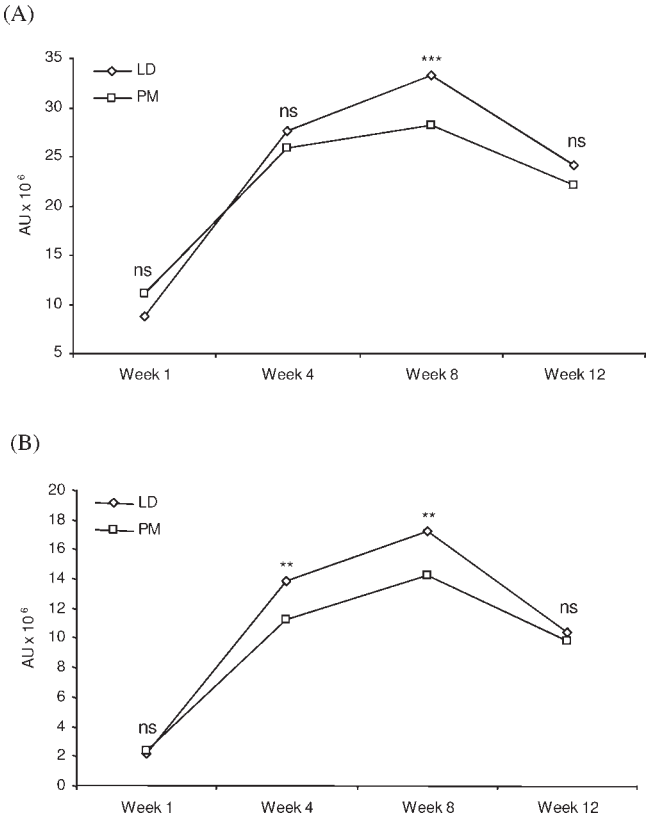

Figure 1. Effect of muscle type (LD vs PM) on the formation of protein carbonyls AAS (A) and GGS (B) at different freezing stages. These results comprise all data from both muscles irrespective of the premincing and packaging technology applied. **, $p < 0.01$; ***, $p < 0.001$; ns, nonsignificant.

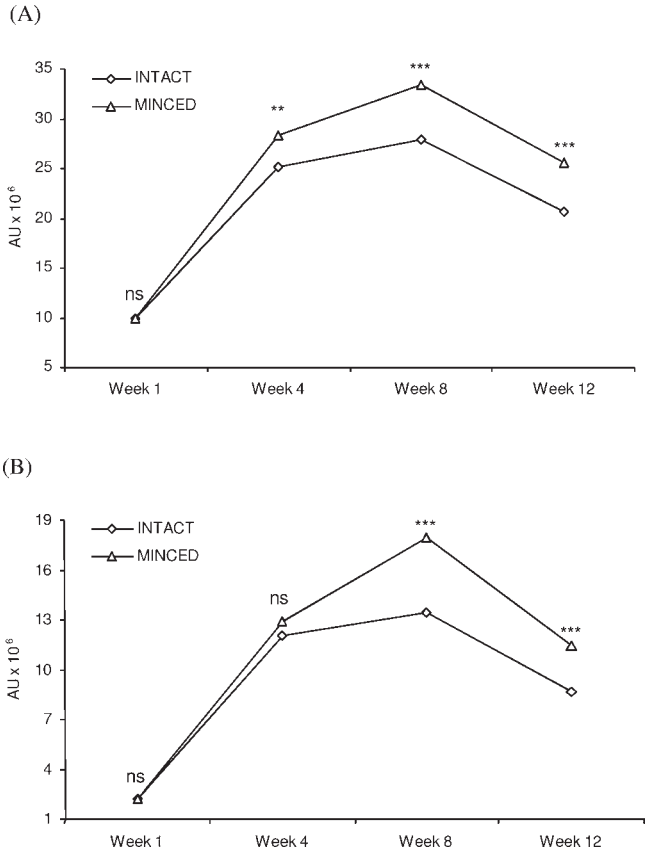

Figure 2. Effect of premincing (MINCED vs INTACT) on the formation of protein carbonyls AAS (A) and GGS (B) at different freezing stages. These results comprise all data from both MINCED and INTACT samples irrespective of the type of muscle and packaging technology applied. **, $p < 0.01$; ***, $p < 0.001$; ns, nonsignificant.

GGS from specific amino acid residues, namely, lysine, arginine, and proline. Previous studies recently reported significant increases of protein hydrazones during frozen storage of fish muscle,[36] pork[8] and poultry.[9] The detection of specific protein carbonyls by using LC-MS avoids certain drawbacks of the DNPH method employed by the aforementioned authors, such as the overestimation of the total carbonyls by accounting for malondialdehyde and other lipid-derived carbonyls attached to proteins through various linkages.[37] Unlike the spectrophotometric method, the analysis of AAS and GGS provides, in addition, precise information on the fate of particular amino acids during the frozen storage of meat and the involved mechanisms. The oxidative deamination of lysine, proline, and arginine to form AAS and GGS requires the initial abstraction of a susceptible hydrogen atom by radical oxygen species (ROS) and an eventual decarboxylation caused by transition metals.[30] A previous study devoted to the in vitro oxidation of myofibrillar proteins revealed that intrinsic components of meat such as myoglobin and nonheme iron are potent promoters of protein carbonylation.[35] These pro-oxidants could have also played a relevant role as initiators of the oxidative reactions in the present study. Between 10 and 20% of the water of the muscle remains liquid at −18 °C, which serves as the medium for diffusion of all other molecules involved in deterioration reactions.[38] Furthermore, the decrease of the water activity as free water freezes leads to the concentration of solutes in the unfrozen water fraction, which causes, in turn, a more effective collision between reactants.[38] It is worth noting that a significant proportion of the unfrozen water in foods would comprise water molecules bound to polar biomolecules (soluble biopolymers) and additional water layers attracted to bound water.[19,38] In the scenario of frozen meats, myofibrillar proteins would attach a large proportion of unfrozen water in which relevant oxidation promoters such as iron and myoglobin would concentrate and exert their pro-oxidant actions. Interestingly, polar amino acid residues from myofibrillar proteins such as the amino-containing side chains, lysine and arginine, are orientated outward with respect to the bulk water[20] and, hence, exposed to the oxidation promoters, which would eventually trigger their oxidation into the corresponding semialdehydes. The enhanced pro-oxidant effect of iron and myoglobin as a result of the cryoconcentration around myofibrillar proteins would explain the considerably large amounts of AAS and GGS found in the present study in comparison to previous analyses carried out in myofibrillar protein isolates and various meat products.[35,39] For instance, the relative amounts of AAS and GGS in pork muscles subjected to frozen storage for 8 weeks are noticeably higher than those found by Ganhão et al.[39] in cooked pork patties refrigerated for 12 days. In the present experiments, the net production of both semialdehydes increased steadily until the eighth week, whereas a significant decrease was detected by the end of the frozen storage. These results suggest that AAS and GGS might be involved in further reactions, which is in good agreement with previous

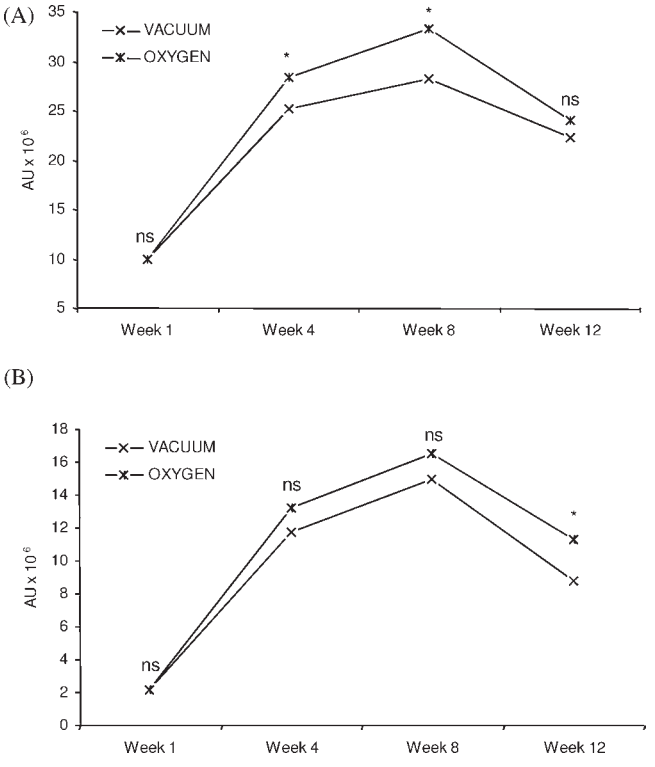

Figure 3. Effect of packaging (VACUUM vs OXYGEN-PERMEABLE BAG) on the formation of protein carbonyls AAS (A) and GGS (B) at different freezing stages. These results comprise all data from both VACUUM and OXYGEN samples irrespective of the type of muscle and premincing technology applied. ∗, $p < 0.05$; ns, nonsignificant.

observations.[23,24,35,39,40] A recent study reported plausible mechanisms by which the aldehyde group of AAS and GGS may be implicated in Strecker-type reactions with free amino acids, highlighting the potential reactivity of the carbonyl moiety of these semialdehydes.[41] Whereas the identification of these carbonyls as oxidation products from myofibrillar proteins is relatively recent,[23] AAS, also known as allysine, has been known in medical research and biological systems for about 30 years. In this field, several papers have reported the high reactivity of the AAS aldehyde group and its participation in aldosamine condensations.[42−44] For instance, Dolz and Heidemann[43] reported that the carbonyl moiety of the AAS molecule reacts promptly with protein-bound lysine as well as with other AAS residues to form Schiff bases and aldol condensation structures, respectively. The occurrence of cross-links between protein semialdehydes and other amine-containing amino acid residues would explain the decrease of AAS and GGS by the end of the storage in the present study. This extent, however, should be supported by future experiments.

The muscle type had a significant effect on the formation of AAS and GGS in pork subjected to frozen storage (Figure 1). The differences between LD and PM were particularly remarkable at the eighth week. In contrast to the results obtained in the unfrozen samples (Table 1), LD had significantly higher amounts of both semialdehydes than PM. The different susceptibility of LD and PM to undergo oxidative reactions during frozen storage may be ascribed to the variations between muscles for their composition in red (oxidative) and white (glycolytic) fibers, which largely determines, in turn, the structure and overall

Table 2. Statistical Effects of Muscle Type, Premincing, Packaging, and Interactions on Measured Parameters

	cooking yield	WHC[a,c]	WHC[b,c]	AAS[c]	GGS[c]
muscle type (M)	ns	**	**	ns	ns
premincing (PM)	ns	**	ns	**	*
packaging (P)	ns	ns	**	*	ns
M × PM	ns	ns	ns	ns	ns
PM × P	ns	ns	**	ns	ns
M × P	ns	ns	ns	ns	ns

[a] Measured according to the sausage formulation technique. [b] Measured according to the centrifugation technique. [c] *, $p < 0.05$; **, $p < 0.01$; ns, nonsignificant.

chemical composition of the muscles.[33,45,46] As aforementioned, numerous previous studies support that muscles rich in oxidative fibers are, in general, more prone to suffer oxidative deterioration than those rich in glycolytic ones.[32,33] In accordance, Soyer et al.[9] recently found larger amounts of protein carbonyls in dark poultry muscles than in light ones subjected to frozen storage (−18 °C/6 months). Lauridsen et al.,[46] however, reported higher lipid oxidation rates in porcine LD than in PM, which is in agreement with protein oxidation measurements from the present study. Taking into account the specific factors and mechanisms involved in the formation of AAS and GGS, a more intense carbonylation was expected in proteins from muscles rich in heme and nonheme iron, such as PM, than in proteins from LD muscles. Beyond the concentration of pro-oxidants, some other influential factors could be responsible for the higher susceptibility of proteins from LD to oxidative reactions. Fiber typing and the metabolic profile of the muscles largely determine the post-mortem biochemical changes and the susceptibility of muscle proteins to suffer denaturation phenomena.[47] In this sense, muscles rich in glycolytic fibers, such as LD, are more prone to undergo a faster pH decline and a more intense denaturation process than muscles rich in oxidative ones.[47,48] Glycolytic muscles, in addition, are believed to suffer more severe proteolysis post-mortem than oxidative ones.[49] Although the connection between protein denaturation, proteolysis, and Pox is not fully understood, processes affecting the solubility and integrity of muscle proteins could enhance the oxidative instability of such proteins and, hence, promote protein carbonylation. Likewise, Pox has been reported to affect the susceptibility of myofibrillar proteins to undergo proteolysis,[50] and the denaturation of myofibrillar proteins during frozen storage occurs with the formation of aggregates and increased cross-linking[51,52] that may involve Pox phenomena. Also, the properties of water and water−protein interactions may be of importance. According to Puolanne and Halonen,[20] the denaturation of proteins has effects on water−protein interactions, water structure, ion binding, availability of different sites of protein side chains to water and ions, etc. The precise mechanisms implicated in the interconnection between biochemical changes undergone by muscle proteins during frozen storage remain unclear, although it seems comprehensible that such interconnection is unavoidably taking place.[51]

Besides the effect of muscle type, technological operations prior to frozen storage, such as premincing and packaging, had a great impact on the carbonylation of muscle proteins (Table 2). In particular, premincing significantly influenced the progress of AAS and GGS throughout the frozen storage of pork samples

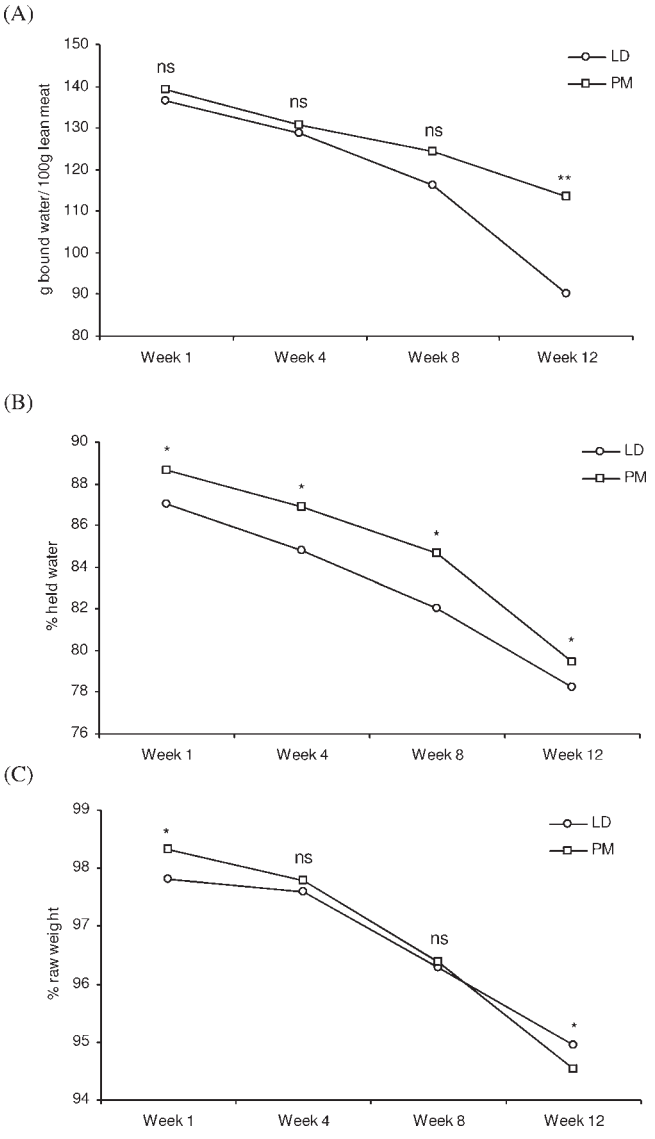

Figure 4. Effect of muscle type (LD vs PM) on the WHC of pork as measured by the sausage formulation (A) and centrifugation techniques (B) and cooking yield (C) at different freezing stages. These results comprise all data from both muscles irrespective of the premincing and packaging technology applied. *, $p < 0.05$; **, $p < 0.01$; ns, nonsignificant.

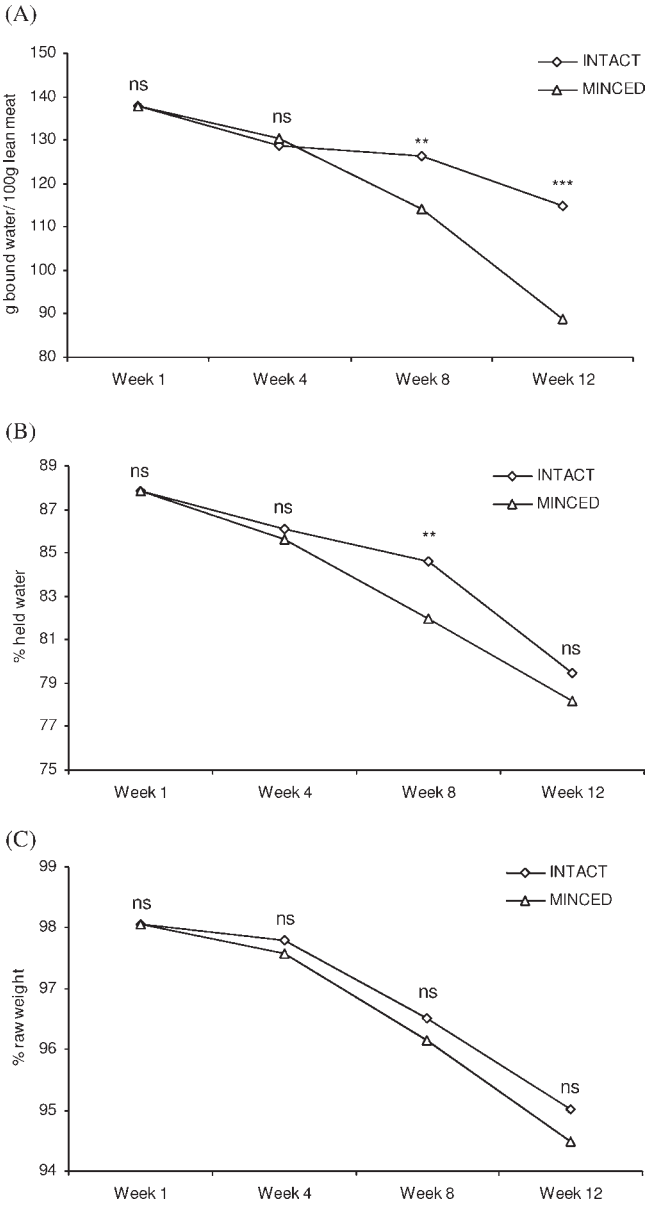

Figure 5. Effect of premincing (MINCED vs INTACT) on the WHC of pork as measured by the sausage formulation (A) and centrifugation techniques (B) and cooking yield (C) at different freezing stages. These results comprise all data from both MINCED and INTACT samples irrespective of the type of muscle and packaging technology applied. **, $p < 0.01$; ***, $p < 0.001$; ns, nonsignificant.

(Figure 2). The differences between MINCED and INTACT samples for the relative amounts of the semialdehydes were significant and particularly noticeable by the end of the storage. According to the present results, premincing of pork muscles enhanced the formation of AAS and GGS during the subsequent frozen storage. These results were expected as technological operations that involve muscle disruption lead to increased oxidative instability. Mincing enhances the interaction between oxidation promoters, ROS, iron, and myoglobin, and the potential targets, lipids and proteins, and increases the incorporation of oxygen into the meat system, leading to larger oxidation rates.[34,48] Other authors previously reported the promoting effect of mincing on the onset and intensity of lipid oxidation during frozen storage of meat and fish.[53,54] The present study confirms the impact of such procedure on the oxidative stability of muscle proteins. Pork packaging was also influential on the evolution of muscle proteins carbonylation during frozen storage (Figure 3). Porcine muscles packaged in oxygen-permeable bags had significantly higher amounts of AAS (weeks 4 and 8) and GGS (week 12) than the vacuum-packaged counterparts. The exclusion of oxygen through the vacuum packaging is generally known to prevent the oxidative degradation of muscle lipids during meat freezing.[55] Unsaturated fatty acids react with molecular oxygen via free radicals and form peroxides or other primary products of oxidation.[34] Molecular oxygen is also implicated in the formation of radicals and peroxides from proteins with susceptible amino acids[10] and, hence, a reduction of protein carbonylation as a result of oxygen exclusion was expected. In fact, Lund et al.[56] previously reported a protecting

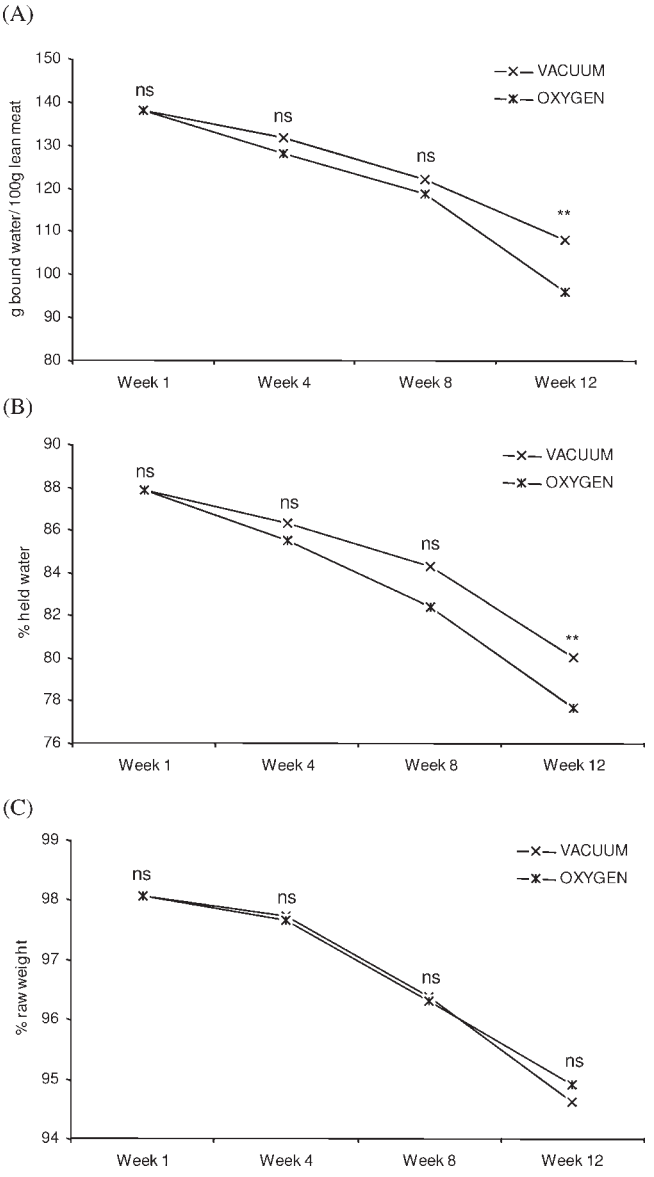

Figure 6. Effect of packaging (VACUUM vs OXYGEN-PERMEABLE BAG) on the WHC of pork as measured by the sausage formulation (A) and centrifugation techniques (B) and cooking yield (C) at different freezing stages. These results comprise all data from both VACUUM and OXYGEN samples irrespective of the type of muscle and premincing technology applied. **, $p < 0.01$; ns, nonsignificant.

effect of anaerobic packaging against Pox in pork patties subjected to chill storage.

Effect of Frozen Storage on WHC. Pork samples corresponding to two different muscles (LD vs PM; Figure 4) and subjected to two different premincing (MINCED vs INTACT; Figure 5) and packaging (OXYGEN vs VACUUM; Figure 6) conditions were stored at −18 °C during 12 weeks to evaluate the impact of frozen storage on their WHC. The WHC of pork samples frozen for 1 week was similar to that displayed by unfrozen and frozen/thawed samples (24 h), but significant decreases were observed in the subsequent sampling times. As expected, the WHC of the porcine muscles decreased gradually throughout the frozen storage in all kinds of samples. Regardless of the technique employed and the pork samples analyzed, the lowest WHC values were found by the end of the frozen storage (week 12). The loss in WHC was from ca. 10 to 30% in 12 weeks, depending on the method of determination (laboratory sausage method or centrifuge method) and studied variable (LD/PM; OXYGEN/VACUUM; MINCED/INTACT).

Table 3. Pearson Correlations between Protein Carbonyl and WHC Measurements

	AAS[a]	GGS[a]
cooking yield	−0.34***	−0.29**
WHC[b]	−0.56***	−0.34***
WHC[c]	−0.60***	−0.34***

[a] **, $p < 0.01$; ***, $p < 0.001$. [b] Measured according to the sausage formulation technique. [c] Measured according to the centrifugation technique.

The WHC of meat is known to be affected by frozen storage, and similar results have been obtained in previous studies devoted to the evaluation of the impact of long-term frozen storage on the technological properties of pork muscles, including WHC.[8,57] According to the existing literature, the loss of WHC in meat as a result of frozen storage is mainly associated with recrystallization phenomena of water and the denaturation of muscle proteins.[2] Taking into account that the WHC of pork as assessed in the present study goes beyond the loss of fluid upon thawing and comprises the ability of finely chopped muscle tissue to bind water within a gel structure, freezing-induced changes affecting the integrity and structure of myofibrillar proteins would play a major role. As reported in most research papers and academic books published during the past decades, the changes related to muscle proteins during frozen storage of meat are proteolysis and freeze denaturation.[2,38] The latter is ascribed to the cooperative impact of the cryoconcentration of solutes and the increase in the area of the ice−water interface during freeze.[2] There will also be a temporal increase of the highly reactive high-density water (HD-water) in the system, due to the lower freezing point of HD-water.[20] The impact that Pox may have on the loss of quality during meat freezing, and particularly on its WHC, has never been considered as the occurrence of oxidative reactions affecting proteins during frozen storage of meat has been largely ignored.

In the present study, positive and significant correlations were found between protein carbonylation measurements and the WHC assessed in pork samples subjected to frozen storage (Table 3). This correlation is supported by the fact that the effects exerted by the muscle type, premincing, and packaging on WHC of pork samples were similar to those aforementioned for Pox (Table 2 and Figures 4−6). For instance, the loss of WHC in LD muscles was more intense than that displayed by PM (Figure 4). The centrifugation technique revealed significant differences between muscles at all sampling times (Figure 4B). In agreement with the Pox results, premincing had a significant effect on WHC as INTACT pork samples had significantly larger WHC by the end of the frozen storage than the MINCED counterparts (Figure 5). In this case, the sausage formulation technique revealed the most remarkable differences between batches. Yet again, the vacuum packaging protected pork samples against the loss of WHC as VACUUM pork samples displayed significantly higher WHC values than OXYGEN samples at week 12 (Figure 6). In summary, the more intense protein carbonylation during frozen storage, the greater the loss of the ability of

pork muscles to bind water. The classical theories about meat freezing and WHC may attribute the present results to the occurrence of protein denaturation and proteolysis. However, the fact that the loss of WHC is concurrent with the progress of Pox and is influenced by the same factors leads to the likely deduction that both phenomena might be linked. In fact, Bertram et al.[21] found that the loss of the ability of myofibrillar proteins to hold water occurred upon their oxidation. These authors, however, did not find a likely molecular mechanism by which protein oxidation would affect the water functionality of myofibrillar proteins. Protein carbonylation involves the loss of amino groups from amino acid side chains that would reasonably lead to the modification of the electronic arrangement and the isoelectric point of myofibrillar proteins. The WHC of muscle proteins may be affected by protein carbonylation as the chemical interaction of polar groups from myofibrillar proteins with water molecules is essential for their binding abilities.[19,20] In addition, protein carbonyls could affect the WHC of muscle proteins through their involvement in the formation of inter- and intramolecular cross-links, which is known to reduce the WHC of meat.[20,59] Liu et al.[22] recently ascribed the loss of WHC in myofibrillar proteins to the formation of intense cross-linking between proteins as a result of the oxidative stress. Interestingly, these authors claimed that unidentified oxidative linkages other than disulfide bonds would be involved in the hydration behavior of myofibrillar proteins. The implication of AAS and GGS in the formation of cross-links in accordance with previous studies[42−44] would explain the decrease of these semialdehydes by the end of the frozen storage and would reinforce their liability in the loss of WHC of porcine muscles. In addition, the oxidation of myofibrillar proteins could influence the occurrence of other phenomena undergone by proteins with negative consequences on WHC. Pox has been reported to promote protein denaturation and insolubilization due to chemical and structural changes.[15] The results from the present study as well as those from recent previous papers[8,9] highlight that Pox may take place simultaneously and interact with other freezing-induced protein changes.

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