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Water Distribution and Microstructure in Enhanced Pork

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Water characteristics and meat microstructure of $NaHCO_3$-enhanced pork were compared with NaCl- and $Na_4O_7P_2$-enhanced pork using low-field proton NMR relaxometry, advanced microscopy techniques, and traditional meat quality measurements. Porcine samples were enhanced at 4 °C for 48 h with sodium salts individually and in the following combinations: (i) 5% NaCl, (ii) 5% $Na_4O_7P_2$, (iii) 3% $NaHCO_3$, (iv) 5% NaCl and 5% $Na_4O_7P_2$, (v) 5% NaCl and 3% $NaHCO_3$, (vi) 5% $Na_4O_7P_2$ and 3% $NaHCO_3$, and (vii) 5% NaCl, 5% $Na_4O_7P_2$, and 3% $NaHCO_3$. Independently of the marinade used, the water-binding capacity was improved, cooking loss was reduced, and the yield was enhanced compared with nonmarinated pork samples. This was also reflected in the water mobility within the samples measured by proton NMR relaxometry. Visualization of samples by confocal laser scanning microscopy (CLSM) revealed salt-dependent microstructural changes in the green pork samples treated with $NaHCO_3$, giving rise to nearly complete disintegration of overall structures. High-resolution visualization by atomic force microscopy (AFM) further suggested that a higher cooking loss in sodium chloride-enhanced samples could be ascribed to less solubilization and higher heat-induced protein denaturation compared with phosphate- and bicarbonate-enhanced samples.

KEYWORDS: Water-holding capacity; marination; marinating; NMR T_2 relaxation; atomic force microscopy; AFM; cooking loss; curing; meat; phosphate

INTRODUCTION

Enhancement technologies have been developed to upgrade meat cuts with respect to water-binding capacity and sensory characteristics of the derived meat products. Traditionally, active water-binding compounds such as sodium chloride, phosphate, sodium lactate, calcium lactate, lactic acid, and calcium chloride have been used in marinades, as they effectively retain water, improve cooking yield, and enhance textural palatability of pork and beef products (*1*–*8*). In general, curing of meat causes transverse expansion of the myofibrils due to the electrostatic repulsion and partially solubilization of proteins, which together promote uptake of water. A sodium chloride concentration of 4.6−5.8% is known to produce maximum swelling of myofibrils and a simultaneous high water uptake (*9*). Moreover, addition of phosphate compounds, particularly pyrophosphate and triphosphate, increases the water-binding capacity of meat. A phosphate concentration of about 0.3% or higher raises the pH of the meat and thereby shifts the isoelectric point. This results in transverse swelling of myofibrils and at the same time promotes extraction of myosin, which in combination retains added water in the final product (*9*). In addition, sodium bicarbonate is known to be a superior marinating agent, which both reduces drip loss and shear force (*10, 11*), improves the yield of enhanced meat (*3*), and furthermore masks undesirable flavors in sow meat (*12*). In contrast to the more commonly used enhancement ingredients, the basic mechanisms responsible for the enhancement properties of sodium bicarbonate are far from understood in detail.

Proton nuclear magnetic resonance (NMR) relaxometry has been successfully applied to study water distribution and water properties in meat (*13*), which are closely associated with the overall protein structure (*14, 15*), and correlations between water-holding capacity (WHC) and T_2 relaxation distribution have been established in meat (*16*–*19*). Recently, it was also shown that proton NMR T_2 relaxation characteristics correlate strongly with salt-induced swelling in pork (*20*). Such a swelling is consistent with the influences

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of pH and ionic strength on proton NMR T_2 relaxation characteristics of extracted myofibrils (*21*).

In continuation of the work by Sheard and Tali (*3*), who studied the effect of salt, tripolyphosphate, and bicarbonate enhancement on the yield and tenderness of cooked pork loin, the aim of the present study was to elucidate the water characteristics and meat microstructure of $NaHCO_3$-enhanced pork compared to traditional NaCl- and $Na_4O_7P_2$-enhanced pork using low-field ^1H NMR T_2 relaxation measurements, confocal laser scanning microscopy (CLSM), and atomic force microscopy (AFM) in combination with traditional meat quality measurements.

MATERIALS AND METHODS

Animals and Sampling. To obtain well-defined raw material for the present mechanistic study of the influence of different enhancement agents on water characteristics and microstructural changes, *M. longissimus dorsi* from a pig, which was offspring of a Duroc/Landrace boar cross-bred with a Landrace/Yorkshire sow, was used. At the time of slaughter, the pig had a live weight of approximately 100 kg. The pig was slaughtered in the experimental abattoir at Research Centre Foulum. The pig was stunned by 85% CO_2 for 3 min, exsanguinated, and scalded at 62 °C for 3 min. Cleaning and evisceration of the carcass was completed within 30 min post mortem. The carcass was split and kept at 12 °C. Within 3 h post mortem, the carcass was transferred to a chill room, where it was stored at 4 °C. Twenty-four hours post mortem pH of the middle part (20 cm) of the right *M. longissimus* was measured (5.66), and the muscle was excised from the carcass and divided into five cuts. From each cut, eight samples with a size of approximately 1 cm × 1 cm × 4 cm were cut and weighed out (weight 1) resulting in a total of 40 pork samples, which were divided into eight groups of five samples containing one sample from each of the five cuts. Seven of the groups were used for subsequent enhancement of the meat samples as described below, while the last group was used as control.

Marinating and Heating of Pork Samples. Seven marinade solutions containing the following were made: 5% (w/w) NaCl (S); 5% (w/w) $Na_4O_7P_2$ (P); 3% (w/w) $NaHCO_3$ (C); 5% (w/w) NaCl and 5% (w/w) $Na_4O_7P_2$ (SP); 5% (w/w) NaCl and 3% (w/w) $NaHCO_3$ (SC); 5% (w/w) $Na_4O_7P_2$ and 3% (w/w) $NaHCO_3$ (PC); 5% (w/w) NaCl, 5% (w/w) $Na_4O_7P_2$ and 3% (w/w) $NaHCO_3$ (SPC). **Table 1** lists the pH of the individual marinades used in the study. Enhancement was carried out on the individual pork samples, which were placed individually in a container with 40 mL of marinade solution. Five replicates were carried out on in each solution. The containers were placed on a vibrating table at a temperature of 4 °C for 48 h. The samples were removed from the marinade solution one by one, dabbed, and weighed out (weight 2). The weight gain as a consequence of processing was determined as the percentage weight gain according to the following equation:

$$\text{Weight gain (\%)} = \frac{\text{weight 2} - \text{weight 1}}{\text{weight 1}} \times 100$$

Moreover, NMR relaxation measurements were carried out at 25 °C on all samples including control samples. Subsequently, the samples kept in closed glass tubes were cooked in a preheated water bath at 70 °C for 20 min followed by temperature equilibrium at 25 °C in a water bath for 20 min. After cooking, the samples were weighed out again (weight 3). Cooking loss and meat yield was calculated by the following equations:

$$\text{Cooking loss (\%)} = \frac{\text{weight 2} - \text{weight 3}}{\text{weight 2}} \times 100$$

$$\text{Yield (\%)} = \frac{\text{weight 3}}{\text{weight 1}} \times 100$$

NMR Measurements and Data Processing. The NMR relaxation measurements were performed at 25 °C on a Maran benchtop pulsed NMR analyzer (Resonance Instruments, Witney, U.K.) with a resonance frequency for protons of 23.2 MHz. The NMR instrument was equipped with an 18 mm variable temperature probe. Transverse relaxation (T_2) was measured using the Carr−Purcell−Meiboom−Gill sequence (CPMG). The T_2 measurements were performed with a τ value (time between 90° pulse and 180° pulse) of 150 μs and using a repetition delay of 3 s. Data were acquired as the amplitude of every second echo (to avoid influence of imperfect pulse settings) in a train of 4096 echoes as an average of 16 repetitions. The obtained T_2 relaxation decays were analyzed using distributed exponential fitting analysis (*22*) by means of in-house-made scripts in Matlab. This analysis yields a plot of relaxation amplitude for individual relaxation processes versus relaxation time.

Data Analysis. Principal component analysis (PCA) was carried out in order to detect the main variations in the distributed T_2 relaxation times induced by different experimental conditions. PCA was carried out on the distributed T_2 relaxation times separately for unheated and heated samples. Principal components are linear functions of the original variables and contain the main structured information in the data. The scores of samples can be used to visualize the similarities, differences, and clustering among the samples under different experimental conditions, while the loadings of variables reflect how much each variable contributes to the variation in the data. Statistical analyses were carried out with the statistical analysis system (SAS, 1991), using the PROC GLM procedure. The models included the fixed effect of marinade.

Confocal Laser Scanning Microscopy (CLSM). Cured samples of *M. longissimus* (approximately 10 mm × 4 mm × 4 mm) were cut and embedded in an OCT compound (Tissue-Trek, Electron Microscopy Sciences, Hatfiles, U.S.A.), immediately frozen in 2-methylbutane precooled with liquid nitrogen and stored at −80 °C prior to sectioning. Serial transverse cryosections of 10 μm in thickness were prepared from the frozen muscle using a cryostat (Leica CM 3050 S, Nussloch, Germany). The sections were fixed with acetone. The sections were incubated with Alexa Fluor 488 phalloidin (0.33 μM) in TBS buffer (pH 7.6) for 30 min and then rinsed thoroughly with TBS and mounted with Prolong gold antifade reagent (Molecular Probes). Images were obtained using a laser scanning confocal fluorescence microscope (Bio-Rad Radiance 2100, AGR-3Q AOTF, Hertfordshire, U.K.), attached to a Nikon Eclipse E800 upright microscope. Excitation was with the argon laser beam at a wavelength of 488 nm, while the emission beam was passed through a 500 nm long-pass emission filter.The images were acquired with Nikon 40x (40x/1,3 oil) and 60x plan Apo (60xA/1,40 oil) objectives. Images of representative areas of each sample were recorded at two different magnifications, 40× and 180× (digital zoom).

Atomic Force Microscopy (AFM). Cured, cooked samples of *M. longissimus* (approximately 10 mm × 4 mm × 4 mm) were cut and embedded in an OCT compound (Tissue-Trek, Electron Microscopy Sciences, Hatfiles, U.S.A.), immediately frozen in 2-methylbutane precooled with liquid nitrogen and stored at −80 °C prior to sectioning. Serial transverse cryosections of 10 μm in thickness were prepared from the frozen muscle using a cryostat (Leica CM 3050 S, Nussloch, Germany). Serial sections were transferred to poly L-lysine-coated 24 mm round coverslips, air-dried, then rinsed gently with distilled water and air-dried again. AFM was performed with a JPK Nanowizard II

Table 1. pH of Marinades[a]

	S	P	C	SP	SC	PC	SPC
	NaCl	$Na_4O_7P_2$	$NaHCO_3$	NaCl + $Na_4O_7P_2$	NaCl + $NaHCO_3$	$Na_4O_7P_2$ + $NaHCO_3$	NaCl + $Na_4O_7P_2$ + $NaHCO_3$
pH	6.62	10.29	8.32	9.65	8.07	8.56	8.26

[a] Abbreviations for marination treatments: S, 5% NaCl; P, 5% $Na_4O_7P_2$; C, 3% $NaHCO_3$; SP, 5% NaCl + 5% $Na_4O_7P_2$; SC, 5% NaCl + 3% $NaHCO_3$; PC, 5% $Na_4O_7P_2$ + 3% $NaHCO_3$; SPC, 5% NaCl + 5% $Na_4O_7P_2$ + 3% $NaHCO_3$.

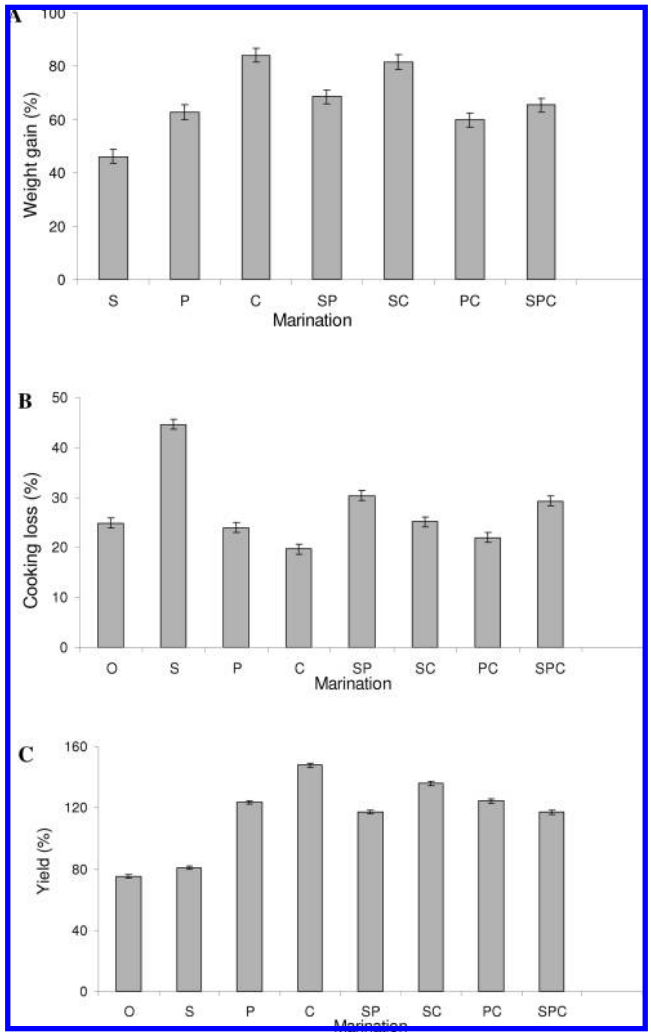

Figure 1. Processing parameters given as (**A**) weight gain (%), (**B**) cooking loss (%), and (**C**) final yield (%) of the pork samples as a function of enhancement procedure. LS Mean values are given. Bars show standard errors.

mounted on a Zeiss Axiovert200 M light microscope and operated in intermittent contact mode at ambient conditions, using a NSG01 cantilever (NT-MDT) with a spring constant of 2.5−10 and a resonance frequency of 115−190 kHz.

RESULTS

Weight Gain, Cooking Loss, and Final Yield. Figure 1 presents initial weight gain, cooking loss, and yield of the pork samples as a function of enhancement procedure. The most pronounced effect was seen in bicarbonate-marinated samples showing highest weight gain and yield and the lowest cooking loss. In contrast, sodium chloride-cured samples resulted in the lowest weight gain and yield and the highest cooking loss. None of the marinade combinations of bicarbonate with sodium chloride and/or phosphate and/or bicarbonate resulted in the same superior processing yield than bicarbonate alone.

Proton NMR T_2 Relaxation. PCA score plots (PC1 versus PC2) of samples before and after cooking, respectively, using the 256 distributed T_2 relaxation times (0.5−3000 ms) as variables are shown in **Figure 2**. For uncooked samples, the explained variance in T_2 distribution by PC1 and PC2 is 87 and 10%, respectively; for heated samples, the explained variance in T_2 distribution by PC1 and PC2 is 70 and 25%, respectively. The score plots reveal that the samples are clustered according to the various enhancement procedures along PC1.

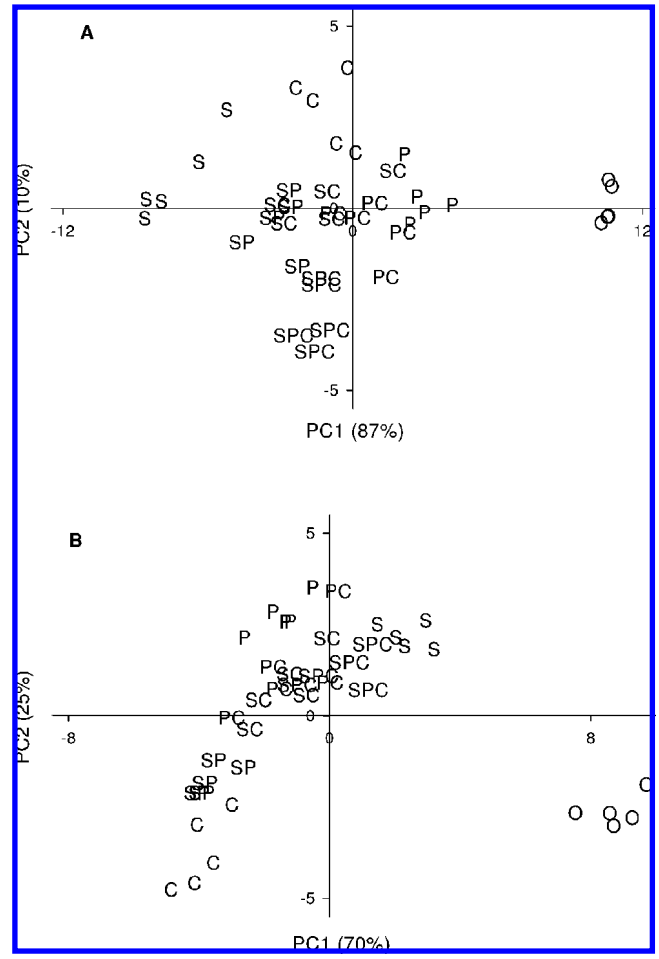

Figure 2. PCA score plots (the first principal component PC1 versus the second principal component PC2) using the distributed T_2 relaxation times as variables for samples (**A**) before heat treatment and (**B**) after heat treatment. For unheated samples, the explained variance in T_2 distribution by PC1 and PC2 is 87 and 10%, respectively; for heated samples, the explained variance in T_2 distribution by PC1 and PC2 is 70 and 25%, respectively.

Figure 3 shows the distributed T_2 relaxation times of fresh meat and samples marinated with NaCl, phosphate, and bicarbonate before and after cooking. The distributed T_2 relaxation times of samples marinated with the combination of the sodium salts were all located within the extremes shown in **Figure 3** and are accordingly not shown. Independent of enhancement agent and cooking, the enhancement of the meat samples shifted the major T_2 component toward the direction of increasing relaxation times. In nonenhanced control samples, the major T_2 component (T_{21}) was centered at 32−75 ms, and a minor T_2 component (T_{22}) was centered at 180−270 ms. Upon enhancement and independent of marinating agent, the relaxation time of the major component (T_{21}) increased to 30−300 ms, hereby resembling a broad distribution covering the area of both the T_{21} and T_{22} components in fresh meat. Noticeably, sodium chloride increased the relaxation times of T_{21} most significantly. The T_{22} component also moved toward increased relaxation times and became less pronounced upon enhancement. After cooking, the control samples were characterized by a broad and asymmetric T_{21} distribution covering the range 15−200 ms. In contrast, the enhanced samples stayed relatively symmetric with regard to their T_{21} distribution and without dramatic changes in relaxation times. However, sodium chloride-cured samples were an exception to this; these samples also tended to display an asymmetric

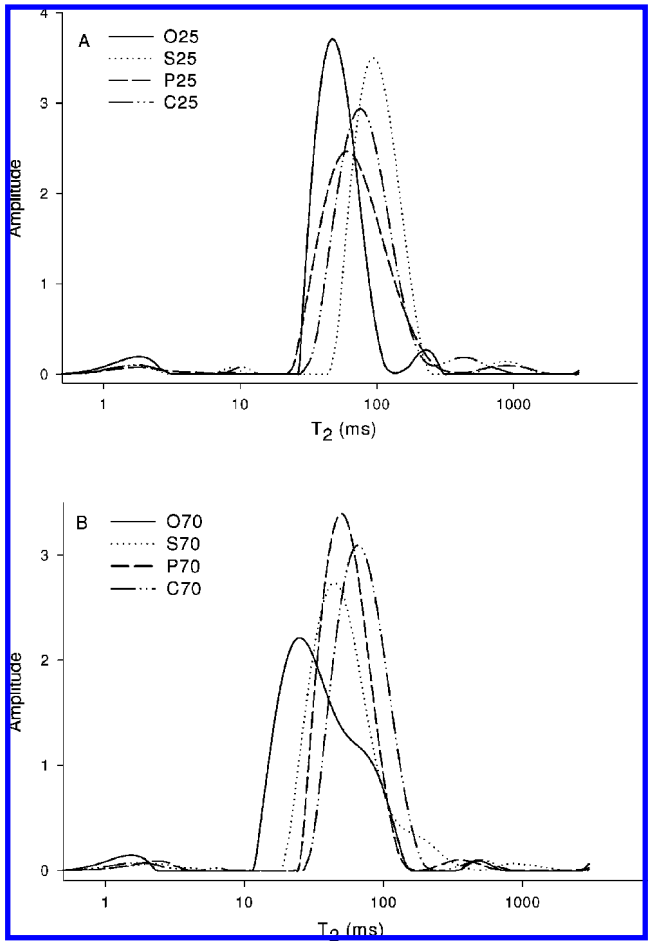

Figure 3. Distributed T_2 relaxation times of the control samples (O), samples enhanced with sodium chloride (S), sodium pyrophosphate (P), and sodium bicarbonate (C): (**A**) before heat treatment; (**B**) after heat treatment at 70 °C. Each curve represents the average of five measurements.

T_{21} distribution. In cooked, marinated samples, the T_{22} component became even less pronounced compared with the uncooked samples.

The mean T_2 relaxation times are shown as a function of enhancement procedure in uncooked and cooked samples in **Figure 4**. Both before and after cooking, the marinated samples were characterized by higher mean T_2 relaxation times compared with the control samples. Before cooking, the highest mean T_2 relaxation time was observed in NaCl-enhanced samples, while this was the case for bicarbonate-enhanced samples after cooking. The reduction in mean T_2 relaxation time upon cooking was highly dependent on the enhancement agent. Bicarbonate enhancement gave rise to the smallest decrease in mean T_{21} relaxation time upon cooking, while sodium chloride enhancement induced the most significant decrease among the samples tested.

Microscopy. The different enhancement agents induced specific changes in the meat microstructure (**Figure 5**). A much more extensive swelling of muscle fibers and subsequently less space between myofibers were observed in bicarbonate-enhanced samples compared with sodium chloride- and pyrophosphate-enhanced samples. In the bicarbonate-enhanced samples, the inherent muscle fiber structure was completely disintegrated and resulted in a substantially different morphology compared to control samples. Sodium chloride enhancement resulted merely in swelling, and samples more or less retained the inherent muscle fiber structure, while the effect of pyrophosphate enhancement was somewhere in between that of bicarbonate and of sodium chloride enhancement.

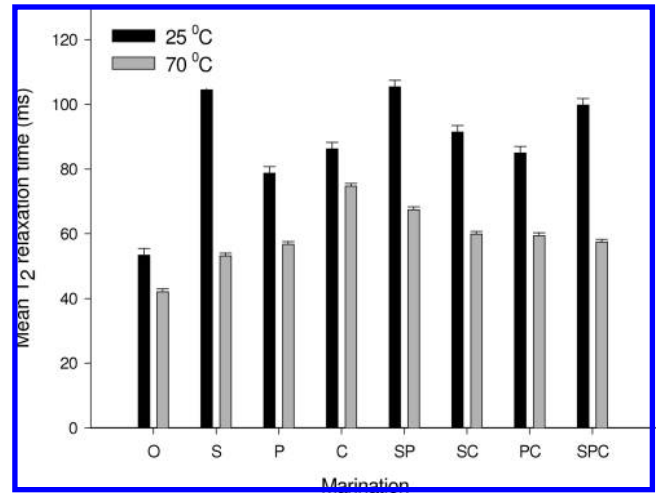

Figure 4. Mean T_2 relaxation time as a function of enhancement procedure for pork samples before heat treatment and after heat treatment. LS mean values are given. Bars show standard errors.

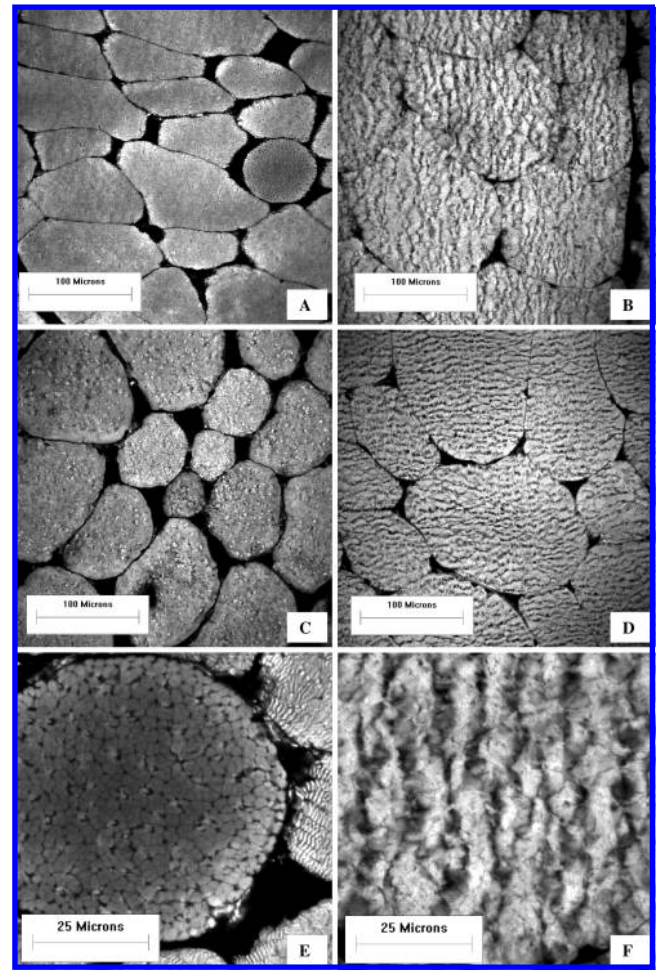

Figure 5. CLSM images of unheated samples: (**A**) control sample, 40× magnification; (**B**) sodium bicarbonate-treated sample, 40× magnification; (**C**) sodium chloride-treated sample, 40× magnification; (**D**) sodium pyrophosphate-treated sample, 40× magnification; (**E**) control sample, 60× magnification; (**F**) sodium bicarbonate-treated sample, 60× magnification.

AFM further detailed the structural effects of the different enhancement agents on cooked samples (**Figure 6**). While the

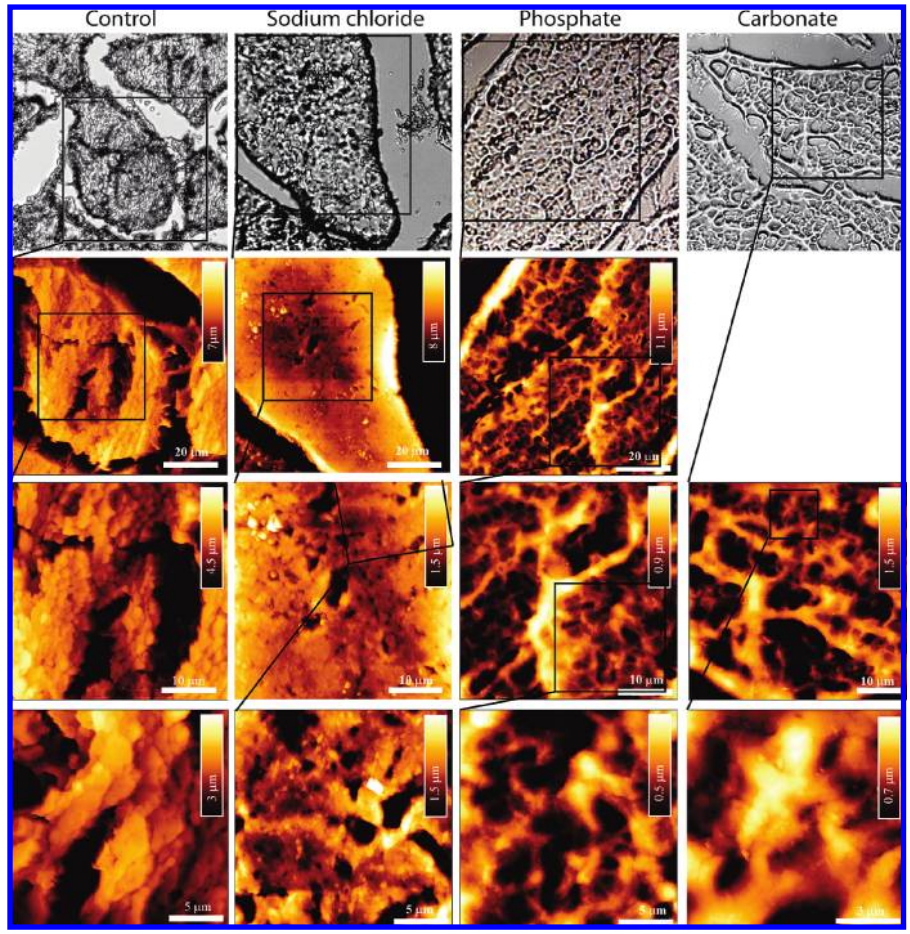

Figure 6. Optical and AFM images of cooked meat samples sliced in 10 μm sections. The marinade used is indicated above each column, and the frame within each image indicates the area scanned in higher resolution in images shown below. The height scale indicated by the color gradient is noted in each AFM image.

bicarbonate-marinated samples appear almost homogeneous in nature, sodium chloride-cured samples were much more heterogeneous with an almost spongelike structure. As observed with CLSM, the effect of pyrophosphate on the meat microstructure was more distinguished than that of sodium chloride; however, the effect was not as dramatic as observed for bicarbonate.

DISCUSSION

Enhancement technologies are of great importance for the meat industry as it allows upgrading of low-value meat cuts. The present study elucidated the effects of different sodium salts on water-binding characteristics, water mobility and distribution, and meat microstructure using traditional meat quality measurements, proton NMR T_2 relaxometry, CLSM, and AFM.

Water Uptake and Distribution. Marinating agents had substantial effects on the water uptake and yield of the meat samples (**Figure 1**). In addition, principal component analyses (PCA) of distributed T_2 relaxation times revealed clear differences in how the various enhancement procedures affected the water mobility and distribution in both green and cooked samples (**Figure 2**). It is worth mentioning that sodium chloride spanned the first principal component, while bicarbonate spanned the second principal component, suggesting different mechanisms behind the two agents' effect on water distribution. In general, the mean T_2 relaxation times (**Figure 4**) increased as a result of marination. The collective effects on water content, protein structure, and the interactions between water and protein molecules are most likely to cause this marinating-induced effect on T_2 relaxation times. An increase in T_2 corresponds to a higher mobility of the water within the meat protein structures due to the higher water content/yield caused by marinating and can be explained by the marinating-induced swelling of the myofibrillar structures (**Figure 5**), which appears to be maintained after cooking in phosphate- and bicarbonate-marinated samples (**Figure 6**). Within the meat protein structures, water molecules are bound by noncovalent bondings (e.g., hydrogen bonds and electrostatic forces), and the individual protein molecules have a variety of electric dipoles, which easily form hydrogen bonds with water molecules. Water molecules in heterogeneous biological systems have been proposed to form polarized multilayers by hydrogen bonds over extended protein surfaces (*23*). Considering this, one of the potential mechanisms of marinating is to expand the exposed surfaces of macromolecules, thereby creating increased interactions between water and the individual meat proteins.

The marinades in the present study had different pH (**Table 1**), and the influence on WHC of this parameter must therefore be considered. It is known that pH affects the WHC by its effect on electrostatic repulsion (*24*), and increases in the pH of meat products using phosphate and bicarbonate salts improve the WHC substantially (*2, 10–12, 25*). Marinating induced a change in meat pH away from the isoelectric point (pH $\cong$ 5.4); however, the present study clearly revealed that the pH of the individual marinades is far from the sole factor determining WHC of the samples (**Figure 1**). If pH is the dominating factor, bicarbonate

marinating should not give rise to superior water-holding characteristics compared with phosphate and combinations of phosphate and sodium chloride, unless the very high pH associated with phosphate gave rise to protein denaturation. Consequently, the specific ingredients also influence WHC as a result of their interactions with the meat proteins. As mentioned previously, phosphate promotes the extraction of myosin (*7*), and previous studies have also shown that phosphate caused dissociation of the actomyosin complex (*26*) and promoted protein extraction from both ends of the A-band (*27*). These effects of phosphate enhance the exposure of protein surfaces and thereby improve the ability of water to form hydrogen bonds with the extended protein surfaces according to the hypothesis given by Ling (*23*).

Meat Protein Microstructure. Sodium chloride-marinated meat took up a considerable amount of water (**Figure 1A**) without any noticeable disintegration of the muscle protein structures (**Figure 5**). This must be expected to occur through the well-known chloride ion-induced weakening of the salt linkages in the muscle proteins, allowing induced swelling and subsequent water uptake (*24*). However, upon cooking, the sodium chloride-enhanced samples lost more water than they gained during the enhancement procedure and thus had inferior yield (**Figure 1C**). This indicates that no pronounced solubilization of the muscle proteins took place during sodium chloride enhancement, which otherwise would have allowed subsequent retention of the water added. In contrast, heat-induced protein denaturation leading to exposure of hydrophobic surfaces (*28, 29*) most probably explains the high cooking loss in sodium chloride-enhanced meat samples. Such denaturation could also explain the pronounced shrinkage as seen by AFM (**Figure 6**).

In contrast to sodium chloride-cured samples, pyrophosphate- and particularly bicarbonate-marinated samples showed higher yields upon cooking. Both pyrophosphate and bicarbonate gave rise to a much higher weight gain than sodium chloride during the enhancement procedure (**Figure 1A**) with bicarbonate being superior. The bicarbonate-induced gain was, as for sodium chloride, followed by considerable swelling (**Figure 5**). However, solubilization of the protein structures upon bicarbonate enhancement was more evident. The high yields in pyrophosphate- and bicarbonate-marinated samples suggest that protein solubilization during enhancement diminished the negative effects of protein denaturation in the subsequent cooking. Solubilization of proteins during enhancement was thus a key difference between samples marinated with sodium chloride versus other compounds. This hypothesis is supported by the more homogeneous water populations in cooked pyrophosphate- and bicarbonate-marinated samples compared to cooked sodium chloride-cured samples, where formation of an asymmetric water population indicates different structural features in the matrix (**Figure 3**). The structural differences were further supported by AFM, which revealed a more homogeneous structure in bicarbonate-marinated samples after cooking, whereas sodium chloride-cured samples had the most heterogeneous structure. The findings are also in accordance with the mean NMR T_2 relaxation times found in the cooked, marinated samples, where the mean NMR T_2 relaxation times in sodium chloride-cured samples decreased severely upon heating, while only limited decrease occurred in bicarbonate-marinated samples (**Figure 4**). These findings suggest less pronounced cooking-induced shrinkage in samples enhanced with bicarbonate compared with other compounds.

The present study clearly demonstrates that solubilization of the muscle protein structures during the enhancement process is critical for a superior yield of heated meat samples. In contrast to the known effect of pyrophosphate on solubilization of meat protein structures (*26, 29*), the effect of bicarbonate on protein structure solubilization is not known, and further studies are needed to elucidate the superior meat protein structure−solubilizing effect of bicarbonate demonstrated in the present study.

Conclusions. In conclusion, the water characteristics and meat microstructure of samples treated with the sodium salts NaCl, $Na_4O_7P_2$, and $NaHCO_3$ either individually or in combinations clearly showed that the chloride, pyrophosphate, and carbonate anion interact differently with the meat proteins, resulting in highly diverse protein structures in both green and cooked pork samples. Bicarbonate seems to be a superior marinating agent resulting in both a high gain in green pork and a high yield in cooked pork. This can be ascribed to the induction of a higher degree of swelling of the myofibrils and a high solubilization effect on meat protein structures, reducing the expulsion of water during cooking. However, further studies are needed to elucidate the basic mechanisms of bicarbonate enhancement and the sensory properties of the marinating agents.

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