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Free Amino Acids, Collagen Solubility, and Meat Quality in Pork (*Longissimus* Muscle of Yorkshire) as a Function of Chiller Temperature and Aging

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Abstract This study was conducted to identify the effect of chilling temperature (−3 and 6°C) and aging (1- and 7-day) on objective meat quality, collagen solubility, and free amino acids in pork (*longissimus* muscle of Yorkshire). Warner-Bratzler (WB)-shear force indicated that variation in chilling temperature had no detectable effect on meat tenderness and tenderization during the 7-day aging period. Among the 13 detected free amino acids, only 3 amino acids (histidine, valine, leucine) were significantly affected by the temperature treatment ($p<0.05$). Collagen solubility was significantly increased at 6°C treatment ($p<0.05$). There was a significant linear relationship ($r=0.67$, $p<0.05$) between changes in free amino acids and WB-shear force during the 7-day aging period. These results confirmed that chilling conditions had significantly affected collagen solubility, and meat tenderization occurred in direct proportion to an increase in free amino acids.

Keywords: Yorkshire, free amino acid, chiller temperature, tenderness, meat quality

Introduction

The most significant single defect of pork meat is the pale, soft, and exudative (PSE) phenomenon, which is generally caused by the loss of water holding capacity (WHC) of muscle fibers leading to soft and pale texture (1,2). Studies have identified that multiple factors are involved in the incidence of PSE meats (3,4) and have also shown that chilling regime is one of prime factors associated with the development of pH related-PSE meat during rigor mortis (5,6). pH and temperature decreases after slaughter, and their interaction during the onset of rigor mortis affect WHC and meat color by impacting protein denaturation and myofibrillar shrinkage (2,7).

Structural and cytoplasmic proteins of meats are exposed to proteolytic actions of endogenous proteolysis during aging and result in polypeptides. The degradation of meats generate small peptides and free amino acids by the subsequent action of peptidases and aminopeptidases, respectively (8). Moya *et al.* (9) showed that changes in free amino acids during aging varied between PSE, RFN (red, firm, and non-exudative), RSE (reddish-pink, soft, and exudative), and DFD (dry, firm, and dark) in pork. These changes are related to aminopeptidase activity under a particular class of pork (8). The results showed that the pH/temperature window during the onset of rigor can have an effect on changes in free amino acids during aging. Cornet and Bousset (10) noted that the accumulation of some free amino acids could result in undesirable flavor and cause decreases in the WHC of the meat. However, free amino acids are of great importance in eating quality due to their specific tastes (11) as their subsequent degradation generates volatile compounds (12).

Depending on the location of pig carcasses within a chiller, the chilling temperature varies from −3 to 6°C. The current study was conducted to identify the effect of chilling temperature and aging on objective meat quality, collagen solubility, and free amino acids in the *longissimus* muscle of Yorkshire.

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Materials and Methods

Animal, experimental design, and treatment Ten market-weighted male pigs (118±13 kg, 194±6.9 day-old) were sampled from the National Livestock Research Institute (NLRI) breeding program. Pigs were transported approximately 3 hr from the research station to the NLRI abattoir by the ordinary commercial trucking system with minimum stress, before being slaughtered the following day, and kept off feed, but given free access to water. The animals, after a low voltage stunning (lower than 280 volts by the mixture of head to head and head to breast technique for 2.5 sec, were conventionally slaughtered, normally hung and alternatively placed in a −3°C (5 pigs), or in a 6°C chiller (5 pigs) until the following day.

Sampling and objective meat quality measurements Tissue samples for free amino acid at 0-hr and relative composition of myosin heavy chain (MyHC)-I isoform in myofibrils were taken during bleeding using a hand-made biopsy machine and immediately stored in liquid nitrogen, and stored at −70°C until analysis. The day after slaughter,

m. longissimus muscles (from the 7th thoracic vertebrae to the last lumbar vertebrae) were removed, halved, and alternatively assigned to 1 of 2 aging periods (1- and 7-day) for objective measurements of Warner-Bratzler (WB)-shear force, drip loss, objective meat color, sarcomere length, and intramuscular fat content. Seven-day old samples were vacuum-packed and stored at 1°C.

Muscle temperature was measured at 5-min intervals for 24 hr (TR-50C; Thermo Recorder, Nagano, Japan) using thermocouples inserted into the *m. longissimus* (geometrical center of the muscle between the 3rd and 4th lumbar vertebrae) from approximately 30 min after stunning, until the following day. The pH was measured by inserting the probe into the center of the muscle between the 3rd and 4th lumbar vertebrae using a portable needle-tipped combination electrode (pH-K21: NWKBinar, Landsberg, Germany) at approximately every 15-min intervals from approximately 30 min post-mortem, until the muscle was judged to have reached ultimate pH. Another measurement was made the following day, approximately 24 hr post-mortem.

WB-shear force was measured on cooked chops (2.54 cm thick) according to the method described by Wheeler *et al.* (13). Sarcomere length at 24 hr was determined using a helium-neon laser diffraction technique according to the method described by Cross *et al.* (14). Drip loss was determined by measuring weight loss of vacuum packed samples after storage for 7 days. Objective meat color was determined by a Minolta Chromameter (CR300; Minolta, Tokyo, Japan) on freshly cut surface after a 30 min blooming at 1°C.

MyHC-I, free amino acid, and collagen content Relative composition of MyHC-I isoform in myofibrils was determined by applying an indirect enzyme-linked immunosorbent assay (ELISA) following Picard *et al.* (15). Briefly, *longissimus* muscle tissue was biopsied during bleeding, immediately frozen in liquid nitrogen, and stored at −70°C until analysis. Crude extracts were made by stirring 200 mg of tissue powder in 1.4 mL extraction buffer (0.3 M KCl, 0.1 M KH_2PO_4, 0.05 M K_2HPO_4, 0.04 M EDTA, pH 6.5, 1 mM DTT) for 15 min on ice. The homogenate was centrifuged at 10,000×g for 20 min at 4°C, and the supernatant was diluted 2-fold with glycerol to a final concentration of 50%, and stored at −70°C until used (16). The protein concentration of the extract was determined as described by Bradford (17), using bovine serum albumin as a standard. Primary and secondary antibodies were human MyHC-I monoclonal antibody (F36.5B9, 2C8, isotype mouse IgG2a; Biocytex Bio- technology, Marseille, France) and rabbit anti-mouse IgG (conjugated with alkaline phosphatase, Bethyl, Lab. Inc., Montgomery, TX, USA). *p*-Nitrophenyl phosphate solution (Sigma, St. Louis, MO, USA) was used for color development and absorbance was measured at 405 nm using a plate reader (MicroScreener LB 9260; EG & E Berthold, Wildbad, Germany). Relative percentage of MyHC-I was calculated against a standard curve for *m. masseter* tissue (15).

Free amino acid levels were determined at 0-hr and 7-day post-mortem, largely according to the method reported by Moya *et al.* (9), but with minor modification. Briefly, 1 g of liquid nitrogen powdered sample was homogenized in 0.01 N HCl with a polytron (3×15 sec) at 4°C and centrifuged at 10,000×g for 20 min. About 300 μL of supernatant was filtered and deproteinized for 30 min at room temperature after mixing with 690 μL of acetonitrile and 10 μL of internal standard (L-citrulline, 250 pmol/μL). Samples were centrifuged at 10,000×g for 15 min and 13 primary amino acids were determined by online derivatization using *O*-phthalaldhyde (OPA). The amounts of free amino acids were analyzed by using the Agilent 1100 HPLC system (Agilent Tech, Waldbronm, Germany) at diode array UV detector (338 nm, 10 nm band wide) with ZORBAX Eclipse-AAA C18 column (4.6×150 mm, 5 μm, Agilent Tech). Separation for each sample was completed in 20 min at 40°C. A gradient mobile phase between 40 mM Na_2HPO_4, pH 7.8 and acetonitrile-methanol-water (45 : 45 : 10, v/v) was used and expressed as pmol.

Heat soluble collagen content determined according to the previously reported methods (18,19). Briefly, 2 g tissue was heated at 105°C for 16 hr and then visualized with 4-dimethylaminobenzaldehyde in perchloric acid (60%, w/v). The reactants were quantified against standard curves of hydroxyproline at 558 nm. The percentage of heat soluble collagen content was determined by calculating the ratio of non-soluble collagen content vs. total collagen content. Two g of homogenized tissue was boiled at 77°C for 70 min and quantification of hydroxyproline were performed same as total collagen content.

Statistical analysis Changes in pH and temperature during rigor development and temperature at pH 6.2 were estimated using a non-linear model (20). The effects of chilling temperature, aging, and their interactions were evaluated by applying a general linear model against a residual error term (21). To determine relationship between changes in free amino acids for 7 days and WB-shear force, a principle component analysis was performed and the first and second components were plotted against changed WB-shear force values (21).

Results and Discussion

Table 1 shows the effect of chiller temperature on carcass traits, the rate of declines in pH and temperature, and objective meat quality traits. The temperature treatments (i.e., −3 and 6°C) did not affect pH at 1- and 24-hr post-mortem. However, carcasses chilled at −3°C resulted in significantly ($p<0.05$) lower temperatures at 1- and 24-hr post-mortem than these placed at 6°C. Consequently, the temperature at pH 6.2 was significantly ($p<0.05$) lower for pigs at −3°C with 15.2 vs. 27.4°C for the 6°C treatment. The result indicated that variation in chiller temperatures ranging −3 to 6°C did not affect the post-mortem glycolytic rate. However, the difference in temperature decline between 2 groups could significantly influence pH/temperature profile during rigor development in the *longissimus* muscle, and consequently could have a significant effect on meat quality traits. Previous studies identified muscle temperature at pH 6.2 as an important threshold to control meat quality because it could be an indirect indication of cold and heat shortening (22), denaturation of myofibril and sarcoplasmic proteins (1), and proteolytic and/or autolytic activity of μ-calpain (23). Geesink and Koohmarike (24) identified that muscle temperature at pH 6.2 had a significant effect on μ-

Table 1. Least square means for the effect of chilling temperature and aging on objective meat quality and collagen solubility

	Temperature (°C)			Aging (day)			F-value	
	-3	6	SE[1)]	1	7	SE	Temp	Aging
Hot carcass weight (kg)	78.4	87.0	5.23	NA[2)]	NA		1.35	
Back fat thickness (mm)	13.2	15.4	1.61	NA	NA		0.93	
Intramuscular fat (%)	1.3	1.8	0.42	NA	NA		0.59	
pH at 1 hr	6.6	6.5	0.10	NA	NA		1.05	
pH at 24 hr	5.7	5.3	0.13	NA	NA		5.85*	
Temperature at 1-hr (°C)	35.7	38.6	0.76	NA	NA		8.39*	
Temperature at 24-hr (°C)	0.1	5.5	0.58	NA	NA		48.2***	
Temperature at pH 6.2 (°C)	15.2	27.4	3.63	NA	NA		6.26*	
MyHC-I (%)	12.3	10.6	1.18	NA	NA		1.14	
Total collagen content (mg/g tissue)	0.5	0.5	0.04	NA	NA		0.20	
Soluble collagen at 7-day (% of total collagen)	18.8	25.5	1.59	NA	NA		9.0*	
Sarcomere length (μm)	1.7	1.8	0.04	NA	NA		1.36	
Drip loss at 7-day (%)	2.7	5.3	0.97	NA	NA		$3.56^{p=0.09}$	
WB-Shear force (kg)	7.6	7.4	0.38	8.9	6.1	0.38	0.13	28.3***
Hunter L*	40.7	44.0	1.10	40.9	43.8	1.10	$4.36^{p=0.052}$	3.44
Hunter a*	6.0	6.1	0.38	5.6	6.5	0.38	0.03	2.95
DF[3)]							1/8 (1/17)	1/17

[1)]Average standard error.
[2)]Not applicable; *$p<0.05$, ***$p<0.001$.
[3)]Numerator/denominator degrees of freedom (where aging term was included).

calpain-mediated proteolysis, but in the current study WB-shear force analysis indicated that the temperature treatment did not affect both meat tenderness and the rate of tenderization during 7-day chiller aging. Numerous studies have reported that completion of rigor mortis at approximately 10-18°C resulted in the most tender meat, and was associated with minimum sarcomere shortening and maximum aging potential (25,26). Given the previous reports more a tender meat for the −3°C treatment was expected, but that was not detectable under the current experimental conditions.

Similarly, sarcomere length did not differ between the treatment groups (1.7 and 1.8 μm for −3 and 6°C, respectively). The current result shows that muscle temperatures ranging from approximately 15 to 27°C at pH 6.2 had an undetectable effect on sarcomere length and meat tenderness. One notable finding was that collagen solubility at 7-day post-mortem was significantly ($p<0.05$) affected by the temperature treatment where carcasses placed in the 6°C chiller. This treatment resulted in significantly ($p<0.05$) higher collagen solubility. Wheeler and Koohmaraie (27) defined connective tissue component as a background toughness, as that changes undetectable level. If that occurred in the Yorkshire *longissimus* muscle studied here, the benefit for tenderness was likely eroded by decreased WHC for samples at the 6°C treatment as drip loss at 7-day post-mortem was significantly higher ($p<0.1$) than those seen in muscles placed at −3°C (Table 1).

Many studies have shown that proteolysis takes place in structural and cytoplasmic protein during aging, and that proteolysis is greatly affected by pH/temperature declines during rigor development as a function of proteolytic and autolytic activities of μ-calpain (23,28). The proteolytic actions of endogenous proteolysis result in polypeptides and the degradation products consequently generate small peptides and free amino acids by the subsequent action of peptidases and aminopeptidases, respectively (8). Despite activities of exopeptidases (i.e., dipeptidylpeptidases and aminopeptidases) significantly varying between pig breeds (29) and muscles (10,30,31), Toldra and Flores (8) suggested that that pH/temperature profile at early post-mortem might affect the rate and extent of aminopeptidase activities.

To understand the effect of chiller temperature on proteolytic actions at the amino acid level we determined content of free amino acids at 0-hr post-mortem (i.e., biopsied sample during breeding) and at 7-day post-mortem. Table 2 shows the effect of chilling regime and aging on free amino acid content. At first glance, all free amino acids measured in the current study was significantly ($p<0.05$) affected by aging time, but only three amino acids (His, Val, Leu) out of 13 amino acids were significantly ($p<0.05$) affected by the temperature treatment. In particular, His and Leu showed significant ($p<0.05$) interactions between chiller temperature and aging time where His and Leu increased more greatly for 6 and -3°C, respectively. There is only limited information available to compare our findings, but the current data likely implies that the chiller temperature affects the level of free amino acids to various extents. Given the fact that free amino acids are of great importance in eating quality due to their specific tastes (11), further studies are likely required to control the level of free amino acids in pig muscles.

Endogenous proteolysis generates small peptides and free amino acids, and the production of these compounds is affected by the interaction between declines in pH and temperature. To further investigate these interaction effects, changes in free amino acids from 0-hr to 7-day post-mortem, and changes in WB-shear force from 1- to 7-day

Table 2. Least square means for the effect of chilling temperature and aging on level of free amino acids (mg/100 g wet tissue)

	Temperature (°C)			Aging (day)			F-value		
	-3	6	SE[1)]	0	7	SE	Temp	Aging	Temp×aging
Asp	0.81	0.73	0.10	0.00	1.54	0.10	0.4	115.0***	
Ser	3.16	2.60	0.34	1.04	4.72	0.34	1.4	59.8***	
His	0.00	5.39	0.30	1.78	3.61	0.30	18.3***	157.6***	18.3***
Gly	5.73	5.08	0.27	3.96	6.85	0.27	2.9	57.0***	
Thr	2.30	2.26	0.25	1.81	2.75	0.25	0.0	7.1*	
Arg	4.00	3.61	0.40	2.10	5.51	0.40	0.5	37.1***	
Ala	68.14	63.92	9.64	13.67	118.4	9.64	0.1	59.0***	
Tyr	7.16	5.62	0.94	3.30	9.48	0.94	1.4	21.7***	
Val	3.66	2.64	0.31	1.94	4.35	0.31	5.3*	29.2***	
Phe	4.04	3.71	0.21	1.79	5.96	0.21	1.2	197.7***	
Ile	3.11	2.90	0.23	1.07	4.94	0.23	0.4	144.0***	
Leu	1.09	5.63	0.28	3.96	2.76	0.28	9.4**	134.5***	4.78*
Lys	4.93	3.75	0.40	1.34	7.35	0.40	4.3	110.6***	
DF[2)]							1/17 (1/16)	1/17 (1/16)	1/16

	Temperature (°C)				
	-3		6		SE
	0-hr	7-day	0-hr	7-day	
His	0.00	3.55	0.00	7.22	0.43
Leu	1.26	6.66	0.92	4.61	0.39

[1)]Average standard error.
[2)]Numerator/denominator degrees of freedom (where the temperature* aging term was included). *p<0.05, **p<0.01, ***p<0.001.

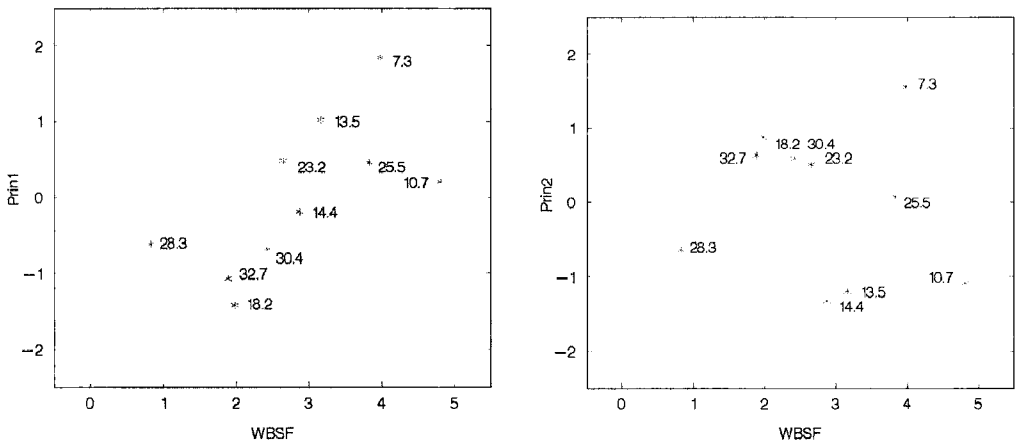

Fig. 1. Relationship between principle component 1 (Prin1) of free amino acids changed from 0-hr to 7-day and WB-shear force (WBSF) changed from 1- to 7-day post-mortem (left figure) and relationship between principle component 2 (Prin2) of free amino acids changed from 0-hr to 7-day post-mortem and WBSF changed from 1- to 7-day post-mortem (right figure). Numbers labelled on each spot are muscle temperatures at pH 6.2. Components 1 and 2 explained 55 and 17% of variations, respectively. Simple correlation coefficients between WBSF and Prin1 and WBSF and Prin2 were 0.67 (p<0.05) and -0.10 (p>0.05), respectively. A significant linear relationship between principle component 1 and WB-shear force suggested that tenderization assessed by WB-shear force was associated with endogenous proteolysis at the amino acid level. On the other hand, no clear relationship between muscle temperature at pH 6.2 and changes in free amino acids or in WB-shear force indicated that the temperature range (e.g., −3 to 6°C) did not affect endogenous proteolytic activity.

post-mortem were compared. Figure 1 shows the relationship between principle components 1 and 2, estimated by including all free amino acids changed from 0-hr to 7-day post-mortem, and WB-shear force from 1- to 7-day post-mortem. The values for each spot represent muscle temperature at pH 6.2 and these were used to determine if the pH/temperature profile during rigor development affected their changes and relationship. There was a significant (p<0.05) linear relationship (r=0.67, data not shown) between principle component 1 and WB-shear force. The results suggested that tenderization assessed by WB-shear force was associated with endogenous proteolysis at the amino acid level. On the other hand, there is no clear relationship between muscle temperature at pH 6.2 and

changes in free amino acids or in WB-shear force. The result indicated that the temperature range (e.g., −3 to 6°C) did not affect endogenous proteolytic activity.

The current study shows that chiller temperature ranging from −3 to 6°C, which could occur in industrial situations, had a limited effect on meat quality traits for the *longissimus* muscle. Tenderization is directly proportional to increases in free amino acid levels. Our findings also indicated that changes in free amino acids occur at various levels at different chilling conditions. Given that free amino acids have a significant effect on taste development, the production and composition of these free amino acids may interact with cooking methods, further studies would prove useful to determine the specific nature of this interaction.

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