IDENTIFICATION OF PROTEOLYTIC PRODUCTS AS INDICATORS OF QUALITY IN GROUND AND WHOLE MEAT

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Accepted for Publication June 7, 1997

ABSTRACT

This study was devoted to the identification of specific peptides and proteins which can serve as indicators of spoilage in meat. Samples of ground and whole meat were subjected to storage at 4C; at intervals of 0, 2, 4, 8, 12 and 16 days, samples were analyzed for pH and microbial populations and subjected to extraction and separation of individual sarcoplasmic and myofibrillar peptides and proteins by SDS (sodium dodecyl sulfate) and native electrophoresis and by RP-HPLC. Sarcoplasmic protein and peptide fractions from RP-HPLC were collected and identified by (electrospray ionization mass spectrometry) ESI-MS. The results demonstrated substantial differences in microbial population and pH between ground and whole meat during storage. Separation by SDS-electrophoresis showed substantial changes in myofibrillar protein of ground meat after 12 days and of whole meat after 16 days of storage. Separation and identification of sarcoplasmic proteins by SDS-electrophoresis and by RP-HPLC followed by ESI-MS revealed the disappearance of a protein fraction band of MW 36 kDa after 8 days of storage in ground and whole meat.

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Journal of Food Quality **21** (1998) 299-316.

INTRODUCTION

Freshness is the most important criterion for judging the quality of meat. Loss of freshness, followed by spoilage, is the result of a combination of complex microbiological, chemical, and physical processes (Pedrosa and Regenstein 1990). Although spoilage in meat is generally associated with microbial activity, deterioration during storage has been attributed to both autolytic as well as bacterial changes; autolytic changes include proteolytic action on muscle and connective tissue (Koohmaraie *et al.* 1988a) and hydrolysis of fat (Anders 1989).

Two endogenous proteinases systems have been proposed to be responsible for proteolytic degradation of myofibrillar proteins (Etherington 1984). The first system is a calcium dependent protease system (CAF, mM-CAF and μM-CAF); these enzymes have maximal proteolytic activities at neutral pH and are capable of degrading minor myofibrillar protein (Schreurs *et al.* 1996). The second system is composed of aspartic proteinases (cathepsin D), cysteine proteinases (cathepsin B, H and L) and cystatins (Ouali 1992); these enzymes have maximal proteolytic activities at low pH and are capable of degrading myosin and actin and a large number of other myofibrillar proteins. Koohmaraie (1992) and Uytterhaegen *et al.* (1994) suggested that CAF was responsible for changes observed during postmortem storage. Mikami *et al.* (1987) and Ouali *et al.* (1987) have shown that lysosomal proteases are responsible for myofibrillar protein degradation. Etherington *et al.* (1987) suggested that there was no relationship between muscle proteolysis and the activity of either CAF or lysosomal enzymes.

Methods for monitoring the changes associated with freshness of meat can be classified as sensory, physical, physico-chemical, chemical and microbiological. Sensory methods are considered the most satisfactory for assessing the freshness of meat; however, sensory methods have some degree of subjectivity. Physical methods include measurement of refractive index, textural changes, electrical conductivity, optical tests, surface tension, viscosity and drip loss; however, these tests have not shown satisfactory correlation with freshness of meat (Pedrosa and Regenstein 1990). Physico-chemical methods which include pH measurement (Seymour *et al.* 1994), the titratable alkalinity (Shelef and Jay 1970), and extract release volume (Patterson and Gibbs 1977), have been proposed, each with its particular advantages and limitations. Microbiological methods used for assessment of microbial quality and safety include standard plate count and selective plate count.

Although there is substantial evidence to suggest that proteolysis is associated in the changes which occur in meat, methods based on monitoring the products of proteolysis as indicators of freshness have received relatively little attention. The identification of a protein fraction of MW 30 kDa as a product of proteolysis of myofibrillar protein extracted from meat has been reported (Negishi *et al.* 1996). Significant developments in techniques for peptide and protein

separation and detection provide the opportunity for more extensive investigation on proteolytic products as indicators of freshness and quality in meat. The objective of the present study was to investigate whether changes in specific protein or peptide fractions can be used as indicators of freshness in ground and whole meat.

MATERIALS AND METHODS

Raw Material and Sample Preparation

A quantity (25 kg) of freshly slaughtered beef (*longissimus dorsi*) was purchased from a local butcher. One half of the meat was ground immediately and identified as ground meat; the other half was cut (approximately 2.5 cm thick × 8 cm in length) and identified as whole meat. The ground meat and whole meat were placed in Styrofoam trays, then wrapped with plastic film and stored at 4 ± 1C. Three samples were taken for analysis after 0, 2, 4, 8, 12 and 16 days of storage.

Microbiological Analyses

Total aerobic (mesophilic and psychrotrophic) and anaerobic bacterial counts were determined at each sampling day according the standard plate count method (AOAC 1990). A quantity (10 g) of ground and of whole meat was placed in sterile stomacher bags (Seward Medical, London, England) containing sterile peptone diluent (90 mL, 0.1%; Difco, Detroit, MI, USA); this mixture was pummelled for 1 min in a stomacher 400 (Seward Medical, London, England) and the homogenate (1 mL) was transferred into a sterile, screw-cap dilution bottle containing peptone diluent (9 mL, 0.1%). Decimal dilutions were prepared for each sample. Mesophilic bacteria counts were determined by placing 1 mL of each dilution in sterile petri dishes. Plate count agar (PCA, Difco, Detroit, MI) heated to 50C was poured into the inoculated plates and the sample distributed by swirling. Psychrotrophic and anaerobic bacteria were determined by spreading 0.1 mL of each dilution on prepoured, dried PCA with a sterile bent glass rod. Anaerobic plates were placed in an anaerobic jar equipped with a palladium catalyst (Fisher Scientific, Montreal, Canada) and carbon dioxide generating kits (BBL, Cockeysville, MD) were used to flush the jar. Incubation of mesophilic and anaerobic plates was done at 37 ± 1C for 48 h while psychrotrophic plates were incubated at 5 ± 1C for 7 days. Colonies were counted after the incubation period.

pH Measurement

pH was measured with a previously calibrated pH meter (Model 750, Fisher Accument, Pittsburgh, PA) by immersing the electrode into meat macerate (25 g) as described by Dhananjaya and Stroud (1994).

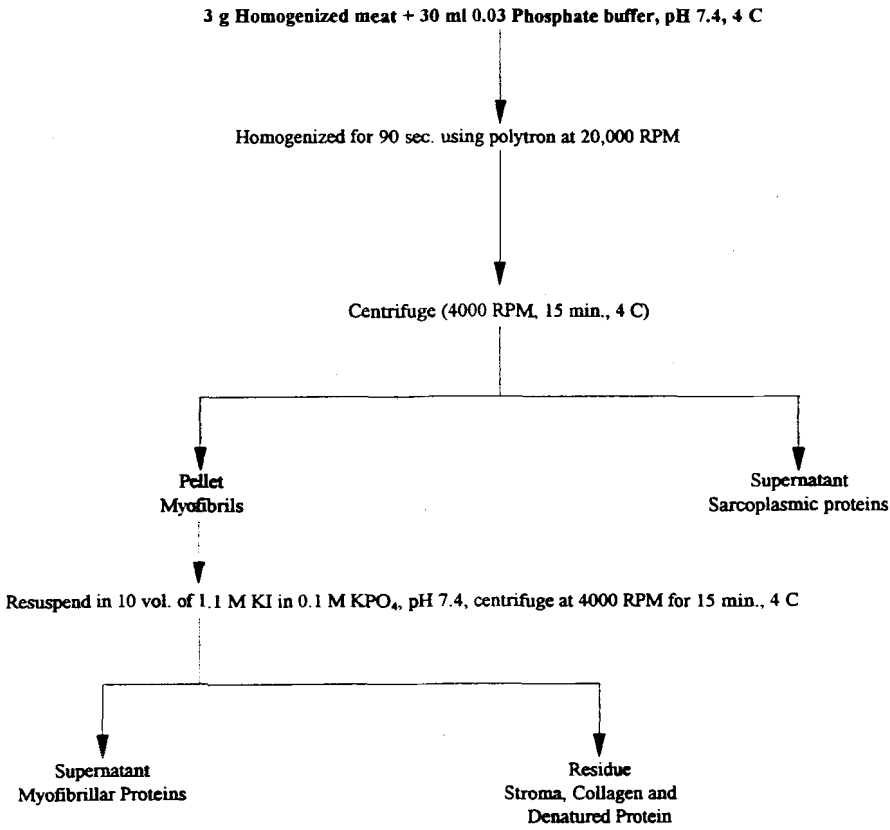

FIG. 1. PROCEDURE UTILIZED FOR EXTRACTING SARCOPLASMIC AND MYOFIBRILLAR PROTEINS FROM MEAT (MOLINA AND TOLDIÁ 1992)

Extraction of Sarcoplasmic and Myofibrillar Proteins

Sarcoplasmic and myofibrillar proteins were extracted from ground and whole meat using the method (Fig. 1) of Molina and Toldiá (1992). All extraction procedures were carried out at 4C. A quantity of meat sample (3 g) was homogenized for 90 s using a Polytron (Polytron^R, PT-MR 3000, Kinematic, AG, Littau, Switzerland) in 30 mL phosphate buffer solution (0.03 M, pH 7.4). Care was taken to prevent the final temperature during blending from exceeding 5C. The mixture was centrifuged at 4000 RPM for 15 min at 4C. The supernatant was filtered through 2 layers of cheese cloth and kept for further analysis. The precipitate (myofibrillar, stroma, collagen and denatured proteins) was resuspended in 10 volumes of 1.1 M KI in 0.1 M potassium phosphate buffer, (pH 7.4). The mixture was blended for 30 s at high speed followed by centrifugation at 4000 RPM for 15 min and the supernatant was retained as the myofibrillar protein fraction. The protein contents of the extracts were measured by the method of Hartree (1972) with bovine serum albumin (BSA) as a standard.

Electrophoresis

Sarcoplasmic and myofibrillar proteins were separated by polyacrylamide gel electrophoresis (PAGE) under native conditions (Davis 1964) and in the presence of sodium dodecyl sulfate (SDS) (Fritz *et al.* 1989). The stacking gel and resolving gel of native electrophoresis were 4% and 8% acrylamide, respectively, and 4% and 12% acrylamide for SDS-PAGE, respectively. Slab gels (0.75-mm thickness) were run at a constant current of 15 mA/gel; the electrophoresis was performed with a Bio-Rad Mini Trans blotting system. The temperature of the electrophoresis unit was maintained at 20C, by immersion the bottom part of the unit in a water bath at 12C. Electrophoresis was terminated when the tracking dye front (bromophenol blue) reached the 1-cm mark at the bottom of the slab. The correlation of molecular weight with relative mobility was obtained with the following standard proteins: myosin (MW 200 kDa), β-galactosidase (116.5 kDa), phosphorylase B (97.4 kDa), serum albumin (66.2 kDa), ovalbumin (45 kDa), carbonic anhydrase (31 kDa), trypsin inhibitor (21.5 kDa), lysozyme (14.4 kDa) and aprotinin (6.5 kDa). Molecular weights (MW) of the specific protein bands were estimated from the log MW vs relative migration distance plot that was generated using the protein standards. After electrophoresis, the gels were removed from glass plates and placed for 2 h in a fixing solution which contained 20% (V/V) methanol, 10% (V/V) acetic acid. Protein bands were stained by immersion of the gel for 18 h in 10% (V/V) acetic acid, 20% (V/V) methanol and 0.1% (W/V) Coomassie Brilliant Blue R250. Destaining was carried out by storing of the gel in the fixing solution until the background color was completely removed. The destained gels were stored in 7% acetic acid until

they were photographed.

HPLC and Mass Spectrometry

Sarcoplasmic proteins were separated by reversed phase-HPLC using a Hewlett-Packard HPLC (Model 1090), C_{18} (0.46 × 25 cm length, Vydac CO., Hesperia, CA) and a diode array, UV-visible detector. The sarcoplasmic protein (1 mL) was centrifuged at 14,000 RPM for 4 min, the supernatant was filtered (0.45 μ, Millipore, Beford, MA) and 50 μL was injected for HPLC analysis. The following two buffer gradient systems were used to elute the sample at flow-rate of 1 mL/min: buffer A, 0.1% trifluoroacetic acid (TFA) in water (V/V, pH 2); and buffer B, 0.1% TFA in acetonitrile (V/V); solvent B was increased linearly from 10-70% over 60 min. The eluate was monitored at 210 nm. The fractionated proteins were collected and dried in a Speed-vac concentrator (Savart, NY) under vacuum. Molecular weight of dried fractions was determined by electrospray ionization mass spectrometry (ESI-MS) (Sciex, Inc., Thornhill, Ontario) according to the procedure described previously (Alli *et al.* 1994). Multiply charged protein ions were generated by spraying the sample solution through a stainless steel capillary held at high potential. The voltage on the sprayer was set at 5.2 kV for positive ion production. The sample was delivered to the sprayer by a syringe infusion pump (Model 22, Harvard Apparatus, South Natick, MA) through a fused silica capillary of 100 μm i.d. The liquid flow rate was set at 1.0 μL/min for sample introduction.

Statistical Analysis

The Statistical Analysis System (SAS Institute Inc., Cary, NC) was used for analysis of least significant difference (LSD) of means using completely randomized block design. Whenever the LSD test was found to be significant, the student - Newman - Keuls' and Tukey's tests were performed between the means to confirm the differences. In all cases, results were considered significantly different when $P < 0.05$.

RESULTS AND DISCUSSION

Bacterial Plate Count

Mesophilic, psychrotrophic and anaerobic bacteria counts of whole and ground meat is shown in Fig. 2 (A and B). The initial counts of both meat samples was considered to be acceptable for fresh meat (Pivnik *et al.* 1976). The

initial mesophilic count (log 3.71) of whole meat decreased significantly ($p < 0.05$) during the first 2 days (log 2.99) of storage then increased significantly ($p < 0.05$) and exceeded $\log_{10}$ CFU/g at day 12 of storage. This initial decrease was not observed in psychrotrophic bacteria. Both mesophilic and psychrotrophic counts increased significantly ($p < 0.05$) after day 4 until the experiment was terminated. The counts of anaerobic bacteria remained relatively low (< log 4) throughout the storage period (Fig. 2A). These results were consistent with the findings of Nortje *et al.* (1990) who reported that anaerobic bacteria were not important in determining the shelf life of aerobically chilled whole meat. In ground meat, initial counts of mesophilic (log 5.68), psychrotrophic (log 4.46) and anaerobic (log 4.88) bacteria increased significantly ($p < 0.05$) throughout the storage period and exceeded $\log_{10}$ 7 CFU/g at day 6 of storage (Fig. 2B). The counts of aerobic and anaerobic bacteria were significantly higher ($p < 0.05$) in the ground meat during the storage period, when compared with whole meat (Fig. 2A and B). Mates (1983) indicated that the grinding process of meat distributes bacteria normally present on the surface of meat throughout the product, and creates ideal conditions for their multiplications. This explains the higher initial count of ground meat. Mackey and Kerridge (1988) indicated that the count of anaerobic bacteria is useful to evaluate the hygiene and public health concern of ground meat. Gill and Newton (1978) indicated that aerobically, there is no inhibitory interactions between different bacterial species until the maximum cell density (10^9-10^{10}) are reached. This was observed in our experiment with ground meat in which both aerobic and anaerobic bacteria counts increased linearly throughout the storage period (Fig. 2B).

pH

Both ground and whole meat had initial pH of 5.70; this is similar to the pH reported previously for ground meat (Seymour *et al.* 1994) and for whole meat (Koohmaraie *et al.* 1988b). Whole meat showed little change in pH during storage, while the pH increased significantly in ground meat after day 4. Seymour *et al.* (1994) reported that changes in meat pH were dependent on the number and metabolic activity of microorganisms present, the amount of glucose and glycogen metabolized, the buffering capacity of the muscle protein and available oxygen.

Changes in Soluble Protein Content Extracted from Whole and Ground Meat

The initial content of sarcoplasmic and myofibrillar protein in ground meat was 4.6 and 10.0 g/L and in whole meat was 4.9 and 9.4 g/L, respectively. Soluble myofibrillar protein content increased in whole meat after day 4 and after

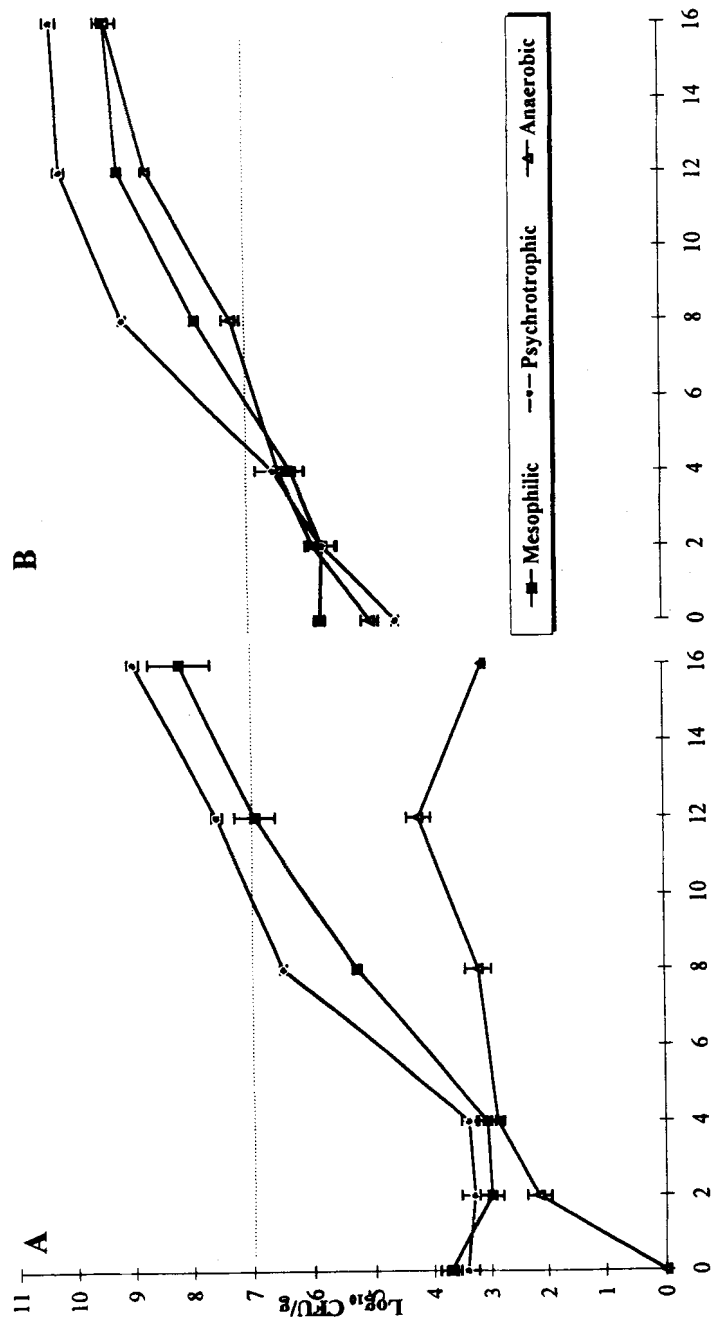

FIG.2. MESOPHILIC, PSYCHROTROPHIC AND ANAEROBIC BACTERIAL COUNT OF CHILLED WHOLE (A) AND GROUND (B) MEAT

day 8 in ground meat; however, this increase was not statistically significant ($p > 0.05$). Salm *et al.* (1983) also reported insignificant changes in total soluble protein during storage. Lopez-Bote *et al.* (1989) reported that the use of sarcoplasmic protein was a useful indicator of meat quality. Several reports have indicated that increased storage time (Miller *et al.* 1980), freezing (Awad *et al.* 1968) and postmortem aging (Lan *et al.* 1993) reduce the extractability of meat proteins. Other reports have indicated that changes in protein content during storage are insignificant (Lin and Park 1996). These conflicting reports demonstrate the difficulty associated with the use of protein solubility as an indicator of quality.

Electrophoresis of Extracted Proteins

Myofibrillar Proteins. Native-PAGE separation of myofibrillar proteins showed no detectable difference between the whole and ground meat. In addition, there was no detectable protein degradation in myofibrillar protein in both meat samples during the storage period.

Figure 3 shows the separation of myofibrillar proteins extracted from whole (A) and ground (B) meat by SDS-PAGE. Substantial changes were observed in ground meat after 12 days of storage and in whole meat after 16 days of storage. Band A (MW = 201 kDa) showed substantial loss in intensity between day 8 and day 12 in ground meat (Fig. 3B) and between day 12 and day 16 in whole meat (Fig. 3A). Associated with this change in band A, is a large increase in the intensity of band B (MW = 188 kDa) at day 16 in whole meat (Fig. 3 A) and at day 12 in ground meat (Fig. 3 B). In ground meat band B showed further decrease in intensity at day 16. This was accompanied with the formation of a band C (MW = 160 kDa, Fig. 3 B). The MW of 201 kDa for band A suggests that this band is myosin heavy chain (MW = 200 kDa; Claeys *et al.* 1995). The MW of 188 kDa for band B and 160 kDa for band C are similar to those reported by Bechtel and Parrish (1983) and An *et al.* (1995) who suggested that these two smaller bands result from autolytic degradation of myosin heavy chain. Our results suggest that degradation of myosin heavy chain occurs between day 8 and day 12 of storage in ground meat and between day 12 and day 16 of storage in whole meat. The degradation was very pronounced after day 16 of storage in ground meat. Changes in bands D (MW 104 kDa), band E (MW 88.5 kDa) and band F (MW 66.5 kDa) were detected in ground meat at the 12 and 16 day storage periods and at the 16 day storage period in whole meat. As far as the authors are aware, there have been no previous reports describing changes to the subunit corresponding to bands D and E. There was a disappearance of band G (MW 47.8 kDa) in ground meat at day 12 and in whole meat at day 16. Associated with the disappearance of band G, was an increase in intensity of band H (MW 42.7 kDa) and band I (MW 39 kDa).

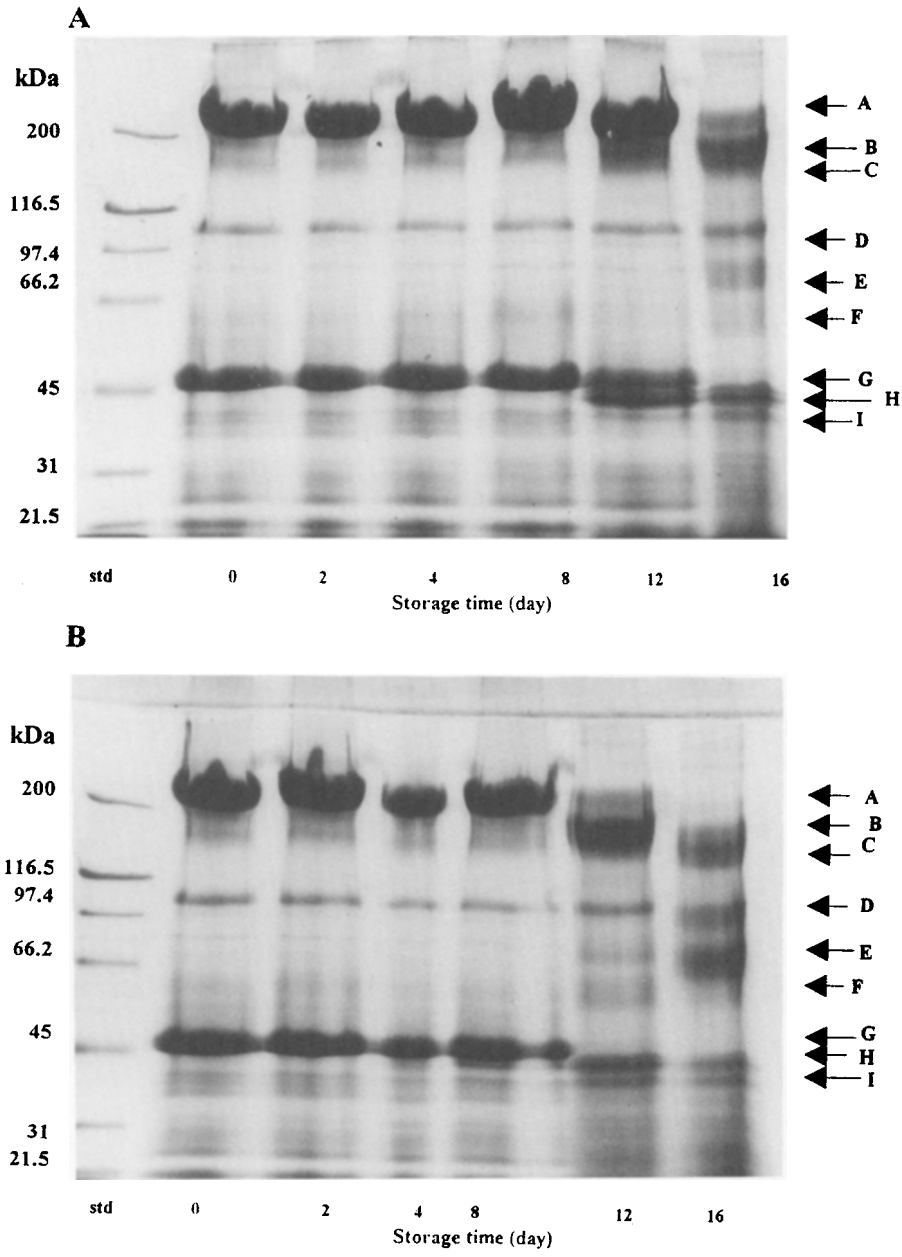

FIG. 3. SDS-PAGE OF MYOFIBRILLAR PROTEINS EXTRACTED FROM WHOLE (A) AND GROUND (B) MEAT AFTER 0, 2, 4, 8, 12 AND 16 DAYS OF STORAGE

It has been proposed that the rapid changes observed in ground meat when compared with whole meat can be attributed to the grinding process (Hamm 1977). Our results suggest that the grinding process which contributed to the high initial microbial load in the ground meat did not affect myofibril proteolysis. Crouse *et al.* (1991) reported that most of the changes in refrigerated meat result from autolytic enzymes during the first 14 days of storage. Beyond this period, microbial proteases are responsible for changes in myofibrillar proteins. The differences observed in myofibrillar degradation between ground and whole meat samples at day 12 and day 16 can also be attributed to changes in pH and bacterial population. Cysteine proteases (cathepsin B1, H and L), which have been shown (Etherington 1984) to be active within the pH range of 5.0-6.0 in postrigor muscle, could have contributed to the observed proteolytic changes. However, the effect of CAF enzymes (μM-CAF) cannot be excluded, since they retain as much as 25-28% of their activity at 5C and pH 5.5-5.8 (Koohmaraie *et al.* 1986; Xiong and Anglemier 1989).

Sarcoplasmic Proteins. Native-PAGE separation of sarcoplasmic proteins showed no detectable difference between whole meat and ground meat. In addition, there was no detectable protein degradation in sarcoplasmic protein in both meat samples during the storage period. Similar results were observed by myofibrillar protein separated under native conditions.

Figure 4 shows the separation of sarcoplasmic proteins from whole (A) and ground (B) by SDS-electrophoresis. Changes were observed in only one band labelled A (MW 40 kDa). This band was not observed at day 12 and day 16 in both whole and ground meat (Fig. 4 A and B). Erickson *et al.* (1983) reported that the CAF enzyme is present in sarcoplasm and have the ability to degrade sarcoplasmic proteins. Our results suggest that these changes were restricted to band A. The fact that the observed changes in the ground and whole meat were similar in spite of the large differences in the microbial population, suggest that the disappearance of band A was not related to microbial activity.

HPLC and Mass Spectrometry of Sarcoplasmic Protein

The chromatograms obtained from HPLC analysis of sarcoplasmic proteins isolated from ground and whole meat is shown in Fig. 5; at least 11 fractions were resolved. The region of the chromatogram in which fractions 4 to 11 were eluted, was similar for samples at the various storage period and for both ground and whole meat. The region of the chromatogram in which fractions 1, 2 and 3 were eluted, showed substantial changes during the first four days of storage in both ground and whole meat. These three fractions were identified by the mass spectrometry. Fraction 3 decreased substantially after day 4 and was almost

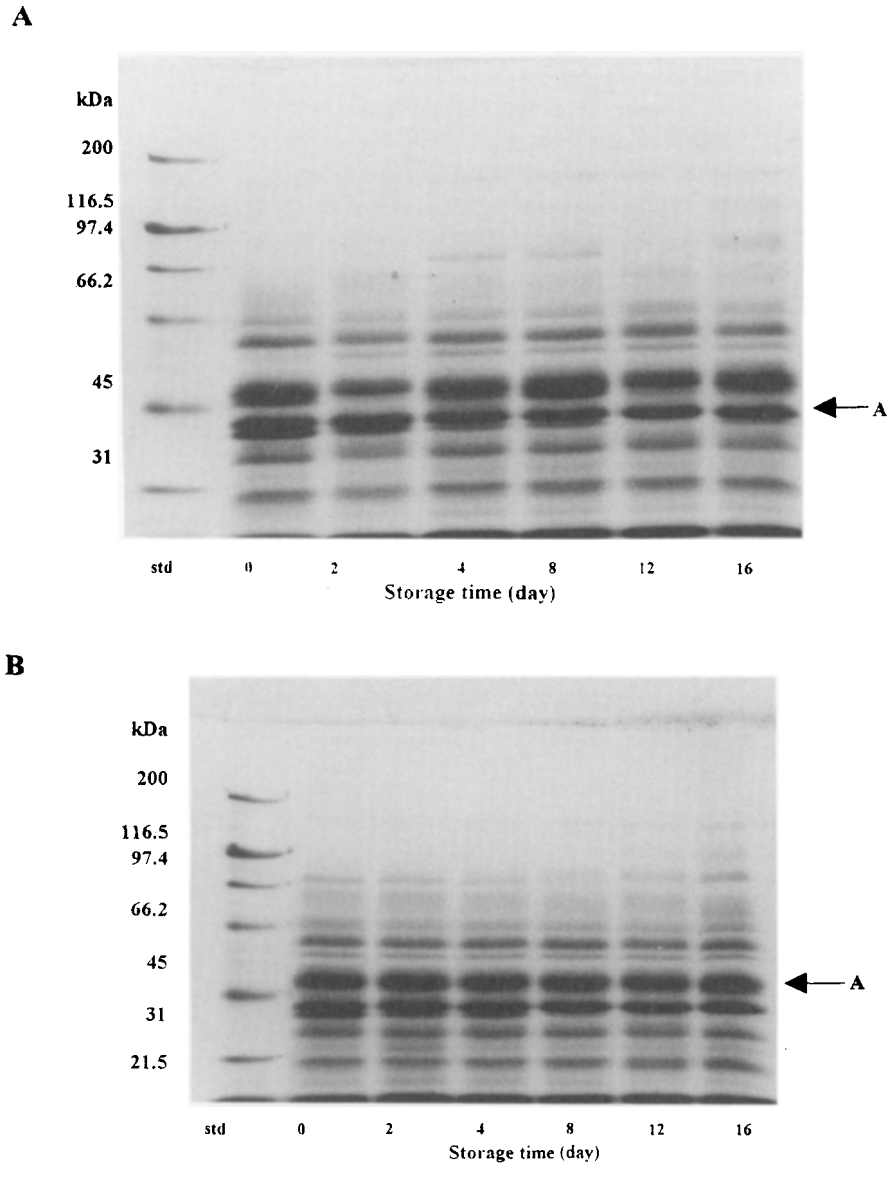

FIG. 4. SDS-PAGE OF SARCOPLASMIC PROTEINS EXTRACTED FROM WHOLE (A) AND GROUND (B) MEAT AFTER 0, 2, 4, 8, 12 AND 16 DAYS OF STORAGE

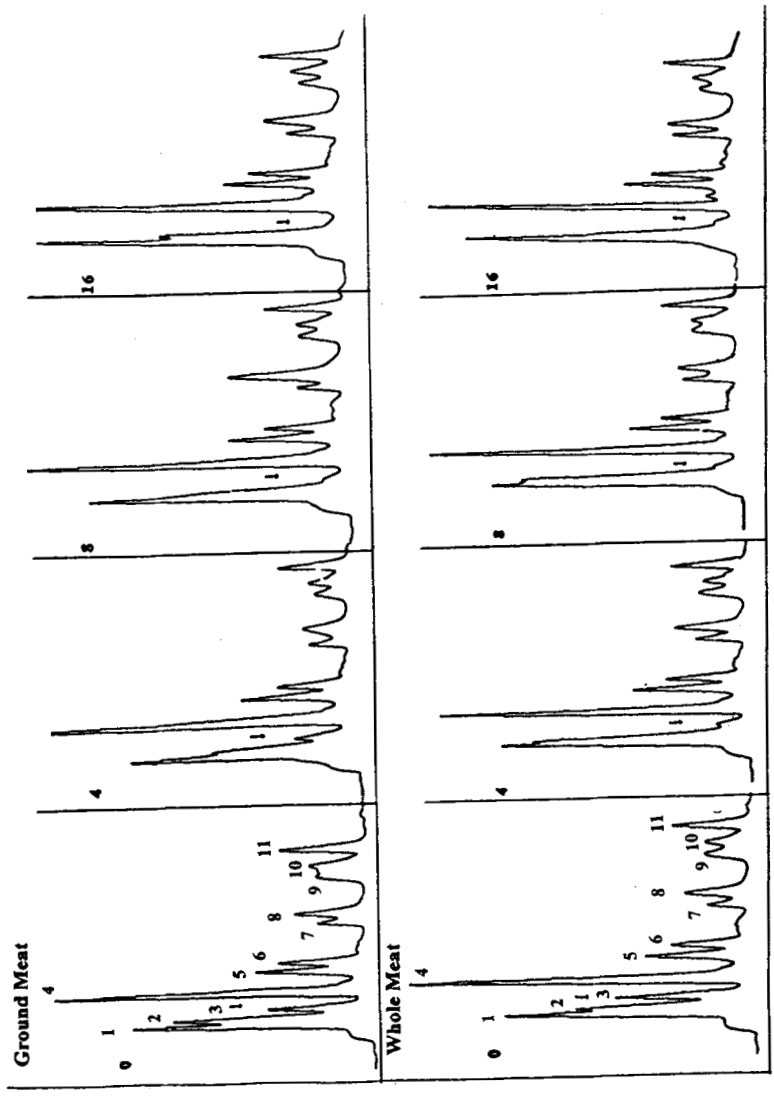

FIG. 5. RP-HPLC CHROMATOGRAM OF SARCOPLASMIC PROTEINS EXTRACTED FROM WHOLE AND GROUND MEAT

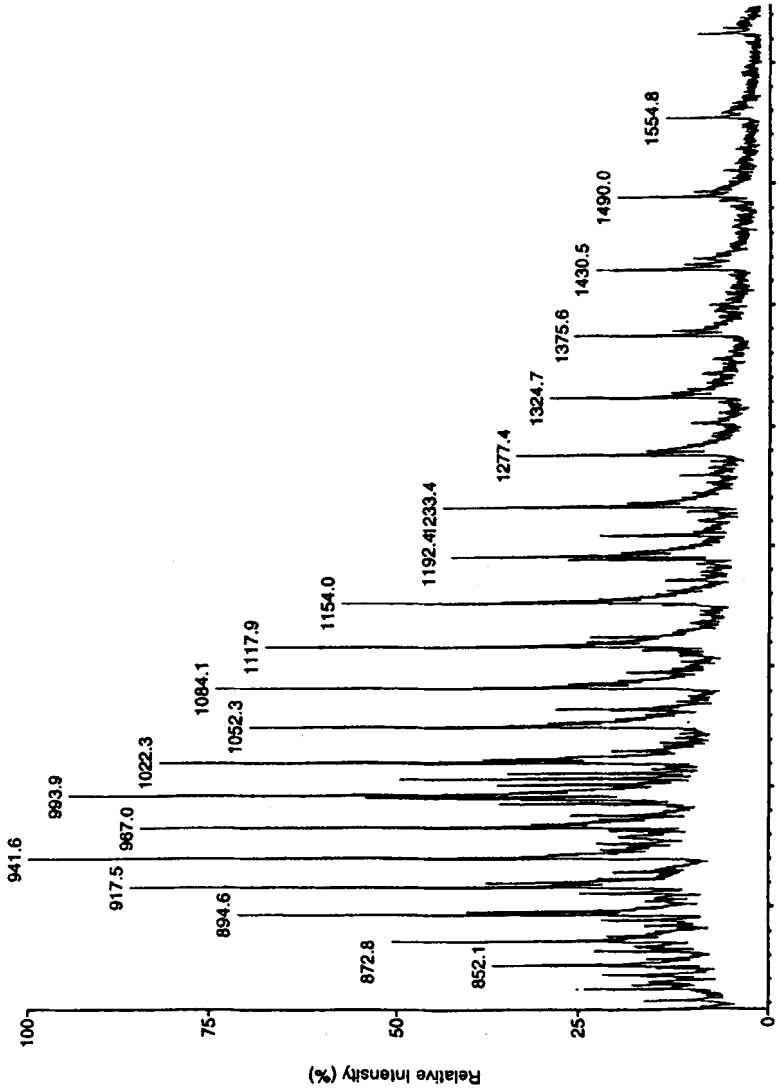

FIG. 6. INTERPRETED MASS SPECTRA OF PEAK 3 FROM THE RP-HPLC OF THE SARCOPLASMIC PROTEIN EXTRACT

undetectable after 8 days of storage of both ground and whole meat. Figure 6 shows the interpreted mass spectra of fraction 3; interpretation of the spectra revealed a MW of 35740 D.

The results from fractionation of the sarcoplasmic by SDS-PAGE and by RP-HPLC followed by ESI-MS both demonstrated a disappearance of a protein fraction after the 8 day storage period of both whole and ground meat. MW of the band which disappeared was determined to be 40 kDa by SDS-PAGE and 35740 D by RP-HPLC/ESI-MS. The similarity between the MWs obtained by the two techniques suggest that both techniques detected the disappearance of the same protein during storage. The actual MW of this protein is the value (35740 D) measured by ESI-MS. Since the disappearance of the band was similar in both ground and whole meat, in spite of the substantially higher microbiological activity in ground meat, it is likely that the disappearance of this protein was the result of autolytic activity. This protein could be investigated as an indicator of freshness.

ACKNOWLEDGMENT

The senior author was the recipient of a postgraduate scholarship from Kuwait Institute for Scientific Research (KISR), Kuwait.

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