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Protease Activity in Post-Mortem Red Swamp Crayfish (*Procambarus clarkii*) Muscle Stored in Modified Atmosphere Packaging

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Protease activity during storage is thought to be an important contributor to decreased shelf life of fresh seafood. To examine this, three batches of red swamp crayfish (*Procambarus clarkii*) tails, placed on trays, were packed with a polyvinyl chloride film (aerobic packaging or AP), under vacuum (vacuum packaging or VP), or under a modified atmosphere (MAP: 80% CO_2/10% O_2/10% N_2), and proteolytic activity was measured on days 0, 1, 3, 6, and 10 during storage at 2 °C. The crude extract from the crayfish digestive system (gut) did not have an apparent role in muscle proteolysis as negligible proteolytic activity was detected. However, the loss of calpastatin (the endogenous calpain inhibitor) was identified in MAP-stored muscle samples on day 10, suggestive of high m-calpain activity. Tail samples stored in AP showed no appreciable proteolysis, but those stored in MAP and VP showed significant decreases in the levels of 53, 66, 71, and 110 kDa polypeptides during storage. The observed proteolytic activity and myofibrillar protein degradation did not correspond to muscle textural properties as the MAP samples had an increased toughness ($P < 0.05$) after storage for 10 days. These findings suggest that other physicochemical mechanisms are involved in postmortem alteration in the crayfish muscle structure under the packaging systems investigated.

KEYWORDS: Red swamp crayfish; modified atmosphere packaging; calpain; proteolysis

INTRODUCTION

Red swamp crayfish, *Procambarus clarkii*, is a freshwater shellfish species native to southeastern United States. It is also known as Louisiana crawfish or Louisiana crayfish. Red swamp crayfish has been aquacultured globally and now can be found in Europe, southeast Asia, central and south America, and Africa. The rapid growth rate and ecological tolerance of red swamp crayfish facilitates a large farming industry in Louisiana and surrounding states (*1*). However, like any other seafood, red swamp crayfish muscle is susceptible to microbial spoilage and hence, must be properly packaged and stored under refrigerated or frozen conditions to preserve shelf life (*2*).

In addition to microbial spoilage, biochemical processes such as protein autolysis caused by muscle endogenous proteases, which were well established for shellfish species (*3, 4*), could have a deleterious effect on swamp crayfish quality, notably deterioration in textural attributes. So far, there is no literature report on the proteolytic changes in swamp crayfish muscle tissue during postmortem storage. Postmortem shellfish muscle tissue is known to be susceptible to endogenous proteases that cause muscle softening and even mushiness. For freshwater prawn, muscle mushiness appeared to be due to the diffusion of proteolytic and collagenolytic enzymes from the hepatopancreas into the muscle tissue (*3, 4*). Calpain, an intracellular neutral endogenous protease, has also been implicated in textural changes in postmortem shellfish muscle (*5, 6*), although the exact role of this enzyme is controversial (*4*).

Fresh shellfish on retail display are traditionally placed on ice unpackaged, or packaged in trays overwrapped with a polyvinyl chloride film. This aerobic packaging condition allows the rapid growth of spoilage microbes. Vacuum packaging is generally not used because of purge loss and the concern for potential anaerobic pathogens. Recently, modified atmosphere packaging (MAP), a storage system that is widely used to curtail microbial growth on red meat, has been introduced to the shellfish industry to extend product shelf life. Using an atmosphere with a high percentage of CO_2 gas, several researchers have shown that MAP can effectively inhibit the growth of

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microorganisms on raw or cooked shrimp (*7, 8*) and red claw crayfish (*9, 10*). Earlier studies also demonstrated the inhibitory effect of MAP on spoilage microorganisms for other forms of crustaceans, including crabs (*11, 12*) and mussels (*13*).

While high-CO_2 MAP was capable of suppressing microbial growth on red claw crayfish when compared with AP, it could significantly alter the physicochemical characteristics of the muscle tissue, notably an increase in toughness presumably from protein aggregation (*10, 14*). In MAP, protein aggregation seemed to counteract proteolysis, which was also promoted by a high concentration of CO_2 (*15*). Ruiz-Capillas and Moral (*16*) reported that in Norwegian lobster kept in a CO_2-rich environment, the loss of free threonine, valine, lysine, and arginine was pronounced, suggesting that protein changes occurred in the muscle tissue.

The mechanism of MAP-induced proteolysis in shellfish has not been studied. The objective of this research was to establish postmortem protein degradation and identify the possible enzymes involved in red swamp crayfish tail muscle packaged in a CO_2-rich atmosphere. Proteases from the crayfish digestive tract as well as calpain and calpastatin from muscle tissue were investigated.

MATERIALS AND METHODS

Sample Preparation. Three-hundred red swamp crayfish (*P. clarkii*), raised at the Aquaculture Research Station of Louisiana State University Aquacultural Research Center (July 4−December 7, 2007) and having a mean tail weight of 7.5 g, were transported live to Kentucky State University. These crayfish were a random mixture of males and females. After being stunned by submersion in an ice slurry for 2 min, red swamp crayfish were manually beheaded. The tails (with exoskeleton on) were placed in iced coolers and shipped to the University of Kentucky's Food Protein Laboratory within 3 h of collection. On receipt, the tails (3 h postmortem) were rinsed with tap water, placed individually (without touching each other) on plastic trays, and then frozen in a −30 °C blast freezer. Frozen tails were then double vacuum packaged and stored in a −82 °C deep freezer and used within 3 months. The temporary freezing was practically necessary for uniform sample packaging treatments. As reported previously, calpains and calpastatin in muscle tissue were remarkably stable during frozen storage at −80 °C for at least 6 months (*17*).

Packaging. The experimental design was a completely randomized factorial structure, and a total of 228 crayfish tails were used for two replicate trials (114 for each). Frozen red swamp tails in double vacuum packages were placed in a 5 °C cooler until they were almost completely thawed (in about 12 h). The tails were placed in 13 plastic trays (32 × 26 × 8 for length × width × depth, respectively, in cm) for the study of proteolytic and textural changes after 0, 1, 3, 6, and 10 days of refrigerated (2 °C) storage. Each tray contained either 5 tails (for days 1 and 6) or 12 tails (for days 0, 3, and 10): 5 tails were used for protease analysis for each of the designated times, and the extra 7 tails for days 0, 3, and 10 were for muscle textural analysis. This arrangement constituted 13 subtreatments in a factorial design with 5 storage times (0, 1, 3, 6, and 10 days) and 3 types of packaging systems representing different atmosphere environments (MAP, VP, and AP); for day 0, only one subtreatment was used.

For AP sample preparation, the trays containing the crayfish tails were double-wrapped with an air-permeable PVC film (15,500−16,275 $cm^3/m^2/24$ h oxygen transmission rate at 23 °C; Bunzl Processor Division, North Kansas City, MO, USA). For MAP, the trays were filled with a gas mixture of 80% CO_2, 10% O_2, and 10% N_2 with a 1.0 bar gas pressure, and packaged with a FoodPack Basic FP372 machine (Ilpra Thermosaldatrici, Vigevano, Italy) using a bilayer polypropylene−polyethylene sealing film (1.75-mil, 140 $cm^3/m^2/24$ h oxygen transmission rate at 23 °C, Amcor Flexibles, Abbotsford, Australia). This MAP gas composition was used because it showed excellent inhibition of microbial growth on red claw crayfish stored at refrigerated temperatures (*9, 10*). For VP, the trays were placed inside type B2620 vacuum bags (2.2-mil, 3−6 $cm^3/m^2/24$ h oxygen transmission rate at 23 °C; Cryovac Division, Sealed Air Corp., Duncan, SC, USA) and packaged using a Model 600A vacuum machine (Sipromac Inc., St-Germain, Quebec, Canada). All packages were stored in a 2 °C cooler. At the end of 0, 1, 3, 6, and 10 days, one pack from each packaging system was removed for analyses.

Detection of Postmortem Proteolysis. Five tails from each packaging system were used for the analysis of proteolytic changes in muscle tissue. After removal of the shell and the digestive tract (gut; for later use), the tails were chopped together, and an appropriate amount of minced muscle was homogenized in 20 mL of 100 mM Tris-HCl (pH 8.3) with 5 mM EDTA to obtain a 2 mg/mL protein concentration. The muscle slurry was mixed (0.33/1, v/v) with an electrophoresis sample buffer (500 mM Tris-HCL, pH 6.8, 20% glycerol, 25 mM EDTA, 25 mM EGTA, 5% SDS, and 500 mM dithiothretol (DTT)) and heated in boiling water (100 °C) for 5 min. The prepared samples were kept in a freezer (−18 °C) until the last samples at the end of day 10 were also prepared, at which time gel electrophoresis and Western blot analysis were performed.

Sodium dodecyl sulfate−polyacrylamide gel electrophoresis (SDS−PAGE) was carried out with a Mini-PROTEAN 3 Cell electrophoresis system (Bio-Rad Laboratories, Hercules, CA, USA), using a 10% acrylamide resolving gel and a 3% acrylamide stacking gel (*18*). An aliquot of 25 μL of sample (1 mg/mL protein) was loaded into each sample well in the stacking gel. A molecular weight (MW) marker, consisting of a cocktail of proprietary recombinant proteins of known MWs (15−250 kDa) (Bio-Rad Laboratories, Hercules, CA, USA), was also loaded. A separate SDS−PAGE was run for the Western blot analysis as described later. Protein bands in red swamp samples were tentatively identified by comparing their electrophoretic patterns with published results of standard muscle proteins (*19*), and MWs of unknown proteins were estimated from the regression line of protein migration distances versus the logarithm MW of the standard proteins.

Activity of Digestive Tract Proteases. Pooled digestive guts were homogenized with 20 mL of 0.1 M phosphate buffer (pH 7.5) with 2 mM DTT using a low-foam homogenizer (Brinkman Instruments, Inc., Westbury, NY, USA). The homogenate was centrifuged (14,000*g*) for 5 min, and the supernatant was used as a crude protease extract. Protein concentration of the extract was adjusted to 2 mg/mL. To determine whether digestive tract proteases might diffuse into muscle tissue thereby contributing to muscle proteolysis, the gut-less tails were chopped together, and an appropriate amount of minced muscle was homogenized in the same 20 mL of 0.1 M phosphate buffer (pH 7.5) with 2 mM DTT as described above.

Proteolytic activity of both digestive tract and muscle extracts was analyzed according to the method of Johnson et al. (*20*). Aliquots of 1 mL of 2% (w/v) azocasein (an enzyme substrate) in glass tubes were mixed each with 3 mL of crude protease extracts. The tubes were incubated in a 30 °C water bath to allow enzyme reactions. An aliquot of 0.5 mL was sampled at 0, 1, 3, 5, and 8 h during incubation and mixed immediately with 0.5 mL of 10% TCA. The mixture was left to stand for 30 min and then centrifuged at 10,000*g* for 10 min. Aliquots of the supernatant (0.7 mL) were mixed with an equal volume of 1 M NaOH. Absorbance was read at 470 nm, and enzyme activity was expressed as absorbance/mg protein.

Activity of the Caplain System. Western blot was performed to detect calpain and calpastatin activity in stored red swamp crayfish tail muscle following the procedure of Guttmann (*21*). After electrophoresis as described above, protein bands on the gel were transferred onto nitrocellulose membranes. The membranes were then placed in a blocking solution containing 1% casein in tris-buffered saline with 0.1% Tween-20 overnight. The treated membranes were reacted separately with primary antibodies diluted with the blocking buffer. For m-calpain, a rabbit monoclonal antibody, RP-1 calpain 2 (Triple Point Biologics, Inc., Forest Grove, OR, USA), was used as the primary antibody, which was diluted 1:5000 and incubated at 4 °C. For μ-calpain, a mouse monoclonal anti-μ-calpain, MA3-940 (Affinity Bioreagents Inc., Golden, CO, USA), was used as the primary antibody, which was diluted 1:4000 and

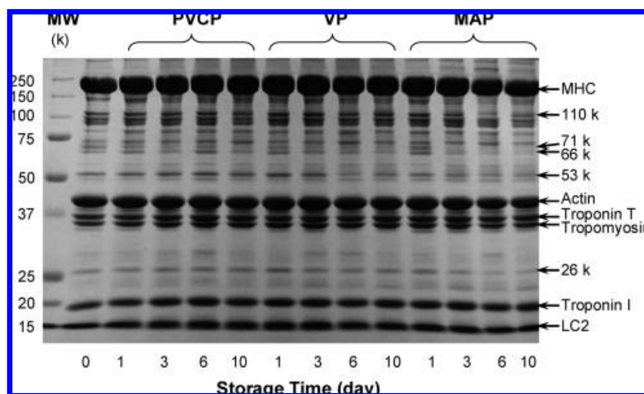

Figure 1. Commassie-stained 10% SDS−PAGE showing differences in muscle protein pattern of red swamp muscle protein stored in three different packaging systems at 2 °C for various time periods. MW, molecular weight (in kDa) marker; MHC, myosin heavy chain; LC2, myosin light chain 2.

incubated at 4 °C overnight. For calpastatin, the mouse anticalpastatin MAB 3084 (Chemicon, Inc., Pittsburgh, PA, USA) was used.

After primary antibody incubations were completed, the membranes were washed three times (10 min/wash) and subsequently reacted with corresponding secondary antibodies (for mouse origin primary antibody, goat antimouse conjugated antibody with horseradish peroxidase was used; for rabbit origin primary antibody, goat antirabbit conjugated antibody with horseradish peroxidase was used). The membranes were developed in 3,3-diaminobenzidine in the presence of hydrogen peroxide, and the images were read with a Model GS-670 imaging densitometer (Bio-Rad Laboratories, Hercules, CA, USA). The results were analyzed using Quantity One software, version 4.5 (Bio-Rad Laboratories, Hercules, CA, USA) as described elsewhere (*21, 22*).

Lipid Oxidation. Lipid oxidation in muscle samples, obtained from the same pooled 5 red crayfish tails used for protease extraction in each experiment replication, was measured by determining the concentration of thiobarbituric acid-reactive substances (TBARS) as described previously (*10*).

Textural Analysis. Seven crayfish tails in each replication were cooked in boiling water (100 °C) for exactly 2 min and then chilled on ice. After removal of the shell, the tails were individually weighed before being subjected to textural analysis using a Model 4301 Instron Universal Testing Instrument (Instron Corp., Canton, MA, USA) with a Warner-Bratzler shearing device attached to the load cell (*23*). The cross-head speed of the Instron was set at 50 mm/min. The maximum force registered in severing the first muscle segment from the anterior of the tail was normalized to the weight (g) of the tail, and the result was expressed as shear force per sample unit weight (N/g) to eliminate the crayfish size effect (*23*).

Statistical Analysis. Data were analyzed using the General Linear Models procedure of the Statistix 7.0 software package (Analytical Software, St Paul, MN, USA) for microcomputers. Analysis of variance (ANOVA) was done to determine the significance of the main effects (packaging treatments; storage time). Significant ($P < 0.05$) differences between means were identified using least significant difference procedures.

RESULTS AND DISCUSSION

Proteolytic Changes. Significant proteolysis occurred in tail muscle samples stored under all three packaging conditions. A prominent feature was the gradual disappearance of the 110-, 71-, 66-, and 53-kDa bands, which, by day 10, were largely decreased from MAP samples and significantly reduced in VP samples (may delete) when compared with the AP system (**Figure 1**), suggesting proteolytic degradation. The 110-kDa component, which is abundant in most invertebrate muscles including those from crustaceans, precisely matched the MW of paramyosin in the white muscle of crayfish (*24*) and hence can be assigned to this protein. The origin of the ∼66-kDa polypeptide, widely shown to rapidly degrade in postmortem crustaceans such as shrimp (*25*) and red claw crayfish (*10, 15*), has not been identified.

The 53-kDa protein could be assigned to desmin (55-kDa) because of their proximity in MW. However, more work is needed to confirm its identity. Desmin is a cytoskeletal protein that functions to link individual myofibrils laterally at their Z disks and interconnect sarcomeres to the sarcomlemma membrane (*26*). The result shown in **Figure 1** indicated that the putative desmin (the 53-kDa protein), along with the 110-, 71-, and 66-kDa proteins, in the AP system was resistant to postmortem degradation but was susceptible to proteolysis in MAP and VP. As AP was a high-oxygen system and the VP and MAP were devoid of or low in molecular oxygen, it is tempting to suggest that postmortem protein degradation was influenced by the redox potential of the packaging systems. Namely, it occurred at a fast rate under anaerobic or low-oxygen conditions and was suppressed in an environment that promoted oxidation.

Several endogenous enzyme systems could potentially contribute to the observed protein degradation. In shellfish, proteases from digestive tracts are considered to be the main causative agents for rapid postmortem degradation and deterioration of muscle tissue (*27*). Calpain and cathepsins are two other proteolytic systems present in muscle tissue, which have been linked to postmortem protein degradation (*6, 28, 29*). Hence, some of these potential proteases were tested in the present study to establish their potential roles in the observed proteolytic changes in red swamp crayfish.

Digestive Tract Proteases. Proteolysis of azocasein by the crude enzyme extract from the digestive tract (gut) of red swamp crayfish was much more intense ($P < 0.05$) compared with the enzyme tract from muscle homogenate in the same packaging system on the same concentration basis (**Figure 2**). Three types of enzymes were found in the digestive tract of larval midgut, two of which were trypsin-like and chymotrypsin-like (*20*). Similar enzyme activity was noted in grass shrimp (*Penaeus monodon*) (*27*). The enzyme activity of the gut extract increased steadily during storage, which was not seen in the muscle extract. The presence of high enzyme activity in the gut but not in the muscle suggested that very little digestive enzymes had diffused into the muscle tissue during refrigerated storage. Thus, their role in postmortem proteolysis in red swamp muscle tissue appeared to be minimal and, in effect, may be ruled out.

Caplain System. The Western blot with anti-m-calpain produced several intense bands, notably those migrating at 80-kDa, 78-kDa (or 76-kDa), and 30-kDa, indicating the existence of m-calpain (or calpain II) in red swamp muscle (**Figure 3**). The 80- and 30-kDa components are the two subunits of calpain. However, the μ-calpain antibody, MA 3-940, did not detect its target (data not shown). Similar results were obtained for lobster claw (*30*) and white muscle of seabass (*31, 32*). For seabass, μ-calpain was only detected during the spawning period (*26*).

Calpain in postmortem muscle tissue is known to be susceptible to autolysis (*28*). It undergoes calcium-dependent autolysis, during which the 80-kDa subunit is converted to a 76-kDa form through a 78-kDa intermediate, and the process was found to be influenced by the redox status of the calpain's environment (*22*). It is clearly visible that the 78-kDa band was partially degraded in muscle samples stored in all three packaging systems. However, the 76-kDa band was observed only in MAP samples at day 10, suggesting a high rate of autolysis or a high m-calpain activity. An increased autolysis

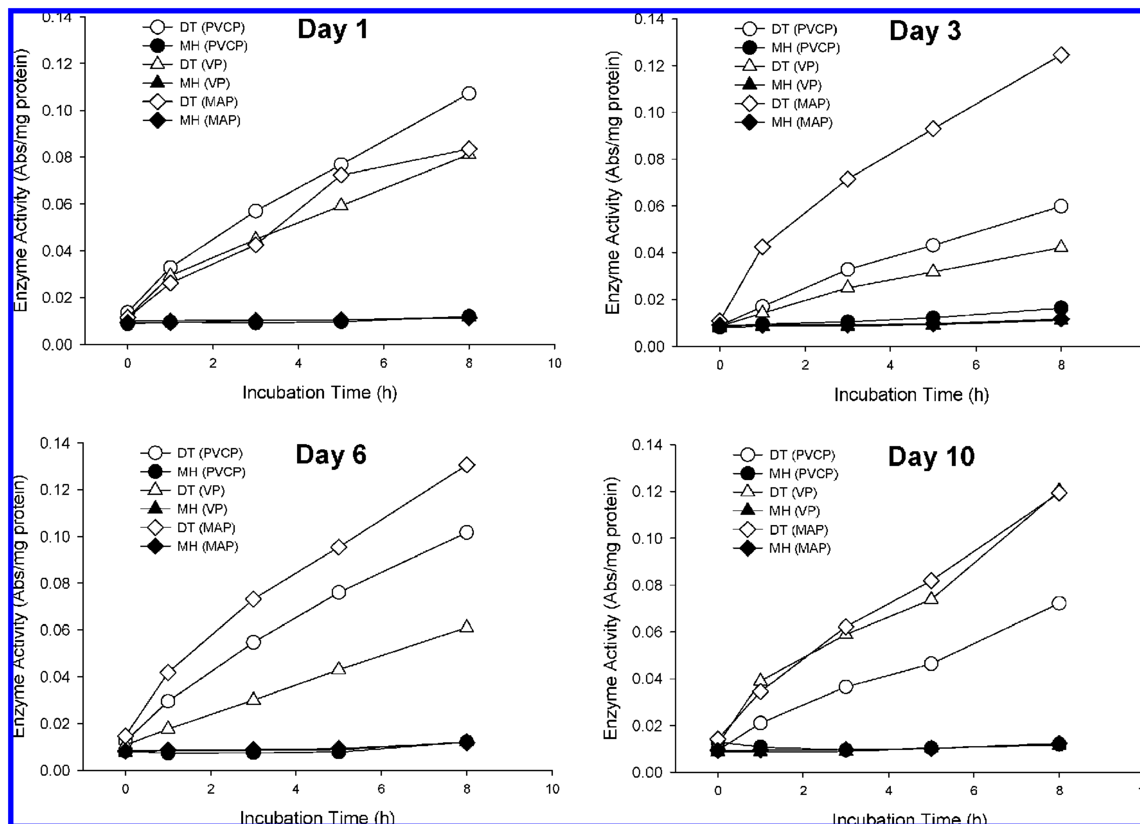

Figure 2. Crude protease activity of extracts from the digestive tract (DT) and muscle homogenate (MH) of swamp crayfish tails stored in AP, VP, and MAP systems for different days. Azocasein (2% (w/v)) was used as the substrate. All of the assays were carried out at pH 7.5 (in 0.1 M phosphate buffer containing 2 mM DTT).

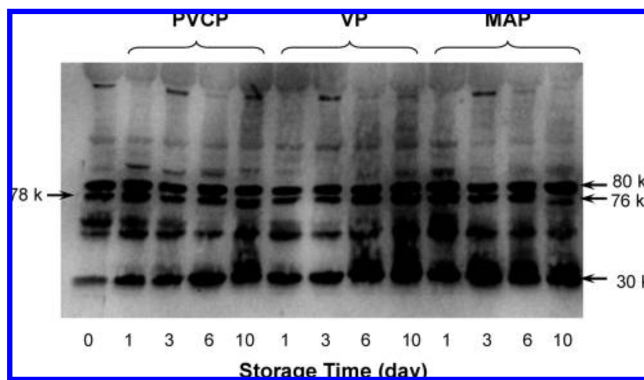

Figure 3. Western blot of m-calpain in swamp crayfish at various time periods in the three different packaging systems using rp1-calpain 2 antibody. The uppermost band is the unautolyzed 80 kDa subunit, the second band is the unautolyzed 78 kDa subunit, and the third band is the autolyzed 76 kDa subunit.

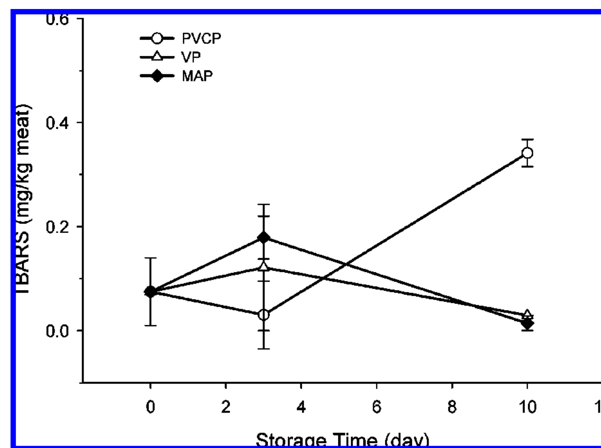

Figure 4. TBARS levels in swamp crayfish muscle stored in three different packaging systems at 2 °C for various time periods. Each data point represents the mean of 3 measurements.

during postmortem storage has been attributed to more calcium being released from the sarcoplasmic reticulum into the cytosol (*33*), which, in the present study, was presumably triggered by the high-CO_2 condition. A rapid autolysis of calpain was well correlated with a high degree as proteolysis in postmortem muscle (*34, 35*). Thus, the apparent fast m-calpain autolysis in the MAP muscle samples, when compared with that in AP samples, seemed to explain the more extensive protein degradation in red swamp crayfish during storage as shown in **Figure 1**.

m-Calpain has an oxidizable cysteine residue at its active site, and it requires reducing conditions to be active (*22*). To determine whether the discrepant m-calpain autolysis in different packaging systems was related to redox conditions, lipid oxidation in stored crayfish samples was monitored. As depicted in **Figure 4**, AP samples exhibited a significantly higher TBARS value than samples stored in MAP and VP on day 10. Therefore, it can be concluded that muscle samples in AP were exposed to a more oxidizing environment and that this could slow down the activation of m-calpain in red swamp crayfish stored in AP. The result supported the findings by Rowe et al. (*35*) who showed that oxidation decreased calpain activity and inhibited myofibrillar protein degradation in postmortem beef muscle.

Calpastatin, the calpain inhibitor, is also widely distributed in vertebrate and invertebrate muscle tissues. The Western blot result of calpastatin showed a positive cross-reaction of anti-calpastatin with the inhibitor (**Figure 5**). However, the intensity

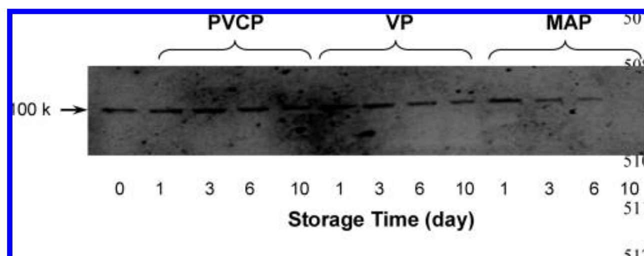

Figure 5. Western blot depicting the degradation of calpastatin in tail muscle extract of swamp crayfish at day 0, 1, 3, 6, and 10 stored in AP, VP, and MAP using MAB 3084 antibody. The lack of a band indicates that calpastatin has been totally degraded.

of the illuminated calpastatin band in MAP samples was reduced rapidly; by day 10, the band completely disappeared, indicating complete degradation. In contrast, no appreciable degradation of calpastatin could be discerned in muscle samples stored in AP. The change in VP was between those of MAP and AP. The loss of calpastatin ostensibly enhanced the conversion of m-calpain from 78 kDa to 76 kDa. The cause for the loss of calpastatin was not clear; it could have resulted from proteolytic degradation. Delbarre-Ladrat et al. (*32*) also found calpastatin to remain constant in fish muscle exposed to air after death. In a recent report, Carlin et al. (*36*) demonstrated that the oxidation of m-calpain with H_2O_2 resulted in a decrease in proteolytic activity and that the addition of calpastatin decreased proteolytic activity even further.

The results from the present study indicated a strong likelihood that the calpain system played a significant role in the observed postmortem red swamp crayfish muscle degradation. However, the overall proteolytic pattern of red swamp crayfish muscle somewhat differed from that of mammalian muscles. For example, troponin T and desmin (53 kDa) in red swamp muscle did not show significant degradation, but in bovine and swine muscles, they are generally the main targets of calpain (*28, 29, 35*). Western blot also failed to detect any troponnin fragments in all red swamp samples (results not shown). The Z disk of crayfish muscle fibers is made up of thin filaments closely packed in parallel arrays and lacks the zigzag characteristic of the disk in vertebrate fibers (*37*). Thus, some cytoskeletal proteins from red swamp muscle might not be fully exposed or accessible by m-calpain. Nevertheless, proteolysis could lead to endomysium detachment in fish muscle (*26*), rendering muscle fibers more susceptible to aggregation.

Muscle Shear Force. The shear force value of AP samples did not change during storage ($P > 0.05$), and that of VP samples initially dropped and then increased (**Figure 6**). However, the shear force for MAP samples continued to increase, and by day 10, it was about 30% higher ($P < 0.05$) than that of the samples in AP and VP. The observed protease activity and myofibrillar protein degradation did not corroborate with this textural property. A similar shear force increase by MAP treatment was seen in red claw crayfish, a related species (*10*). The potential influence of pH can be ruled out as all muscle samples had a pH around 7, independent of packaging systems (result not shown). A number of studies have shown that limited hydrolysis of proteins (e.g., whey and soy proteins) can promote protein aggregation (*38, 39*). Thus, one plausible explanation could be that partial degradation of the muscle myofibril structure and the presumable detachment of muscle fibers that ensued (*26*) would enhance muscle protein aggregation, resulting in higher muscle shear force upon cooking.

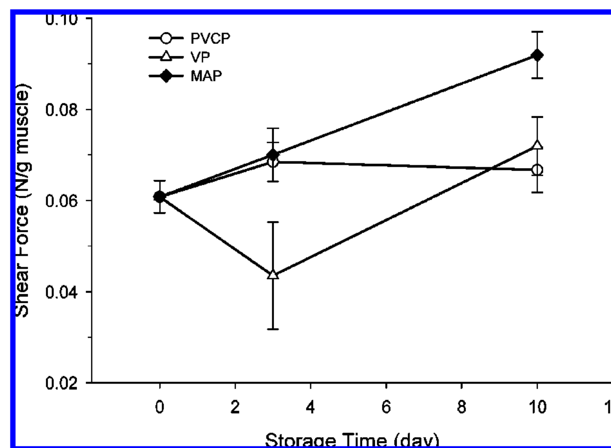

Figure 6. Breaking force of swamp crayfish muscle stored in three different packaging systems at 2 °C for various days. Each data point represents the mean of 7 measurements based on the 7 different samples.

Conclusions. Electrophoretic and immunological analysis of proteolytic changes and protease activity in red swamp crayfish muscle stored in CO_2-rich MAP indicated a lack of measurable contribution of digestive enzymes to muscle proteolysis of red swamp crayfish during refrigerated storage. Rather, the MAP promoted activation of m-calpain and loss of calpastatin, suggesting that calpain was partly responsible for the proteolytic degradation. Further research is needed to determine the molecular mechanism by which the calpain system was regulated by high-concentration CO_2 in the modified atmosphere environment and why proteolysis did not corroborate the observed textural changes in muscle during postmortem storage.

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