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# In Vitro Proteolysis of Myofibrillar Proteins from Beef Skeletal Muscle by Caspase-3 and Caspase-6

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**ABSTRACT:** The objective of the study was to investigate in vitro degradation of myofibrils by caspase-3 or -6. Myofibrillar proteins prepared from beef skeletal muscle were incubated with caspase-3 or -6 at 30 °C for 2 or 12 h, and subsequently, protein degradation was detected. Results showed that caspase-3 and -6 reproduced the degradation patterns of titin and nebulin observed during normal postmortem (PM) aging; however, they only reproduced the 28 kDa fragment derived from troponin-T. Caspase-3 induced only minor degradation of desmin. However, caspase-6 caused increasing degradation of desmin with extended incubation time and produced three degradation fragments (45, 29, and 27 kDa) of which only the 45 kDa fragment has been reported in aged beef. Therefore, caspase-3 or -6 could only reproduce a part of myofibrillar protein degradation or degradation fragments observed in naturally aged meat and may be involved in PM proteolysis of muscle proteins together with other endogenous proteases.

**KEYWORDS:** Caspase-3, caspase-6, myofibrillar proteins, beef muscle, proteolysis, tenderization

## INTRODUCTION

Postmortem (PM) meat tenderization is a complex biochemical process involving the fracturing of key myofibrillar proteins, which are responsible for maintaining the structural integrity of the myofibrils, by endogenous proteolytic enzymes. Several lines of evidence suggest a major role of calpains and especially of calpain 1 or $\mu$-calpain in PM tenderization of meat.[1−3] However, variability in PM tenderness could not be fully explained by calpain system.[4,5] Therefore, it has been recently proposed that the process of meat tenderization results from the synergistic action of several endogenous enzymatic systems. In addition to the well-studied calpain system, the caspase proteolytic system responsible for apoptosis may be a candidate for PM muscle cell degradation in the initial stages of the conversion of muscle to meat.[6]

The caspases are a family of cysteine proteases that play a central role in cells for apoptosis. Caspases implicated in apoptosis are generally divided into two functional subgroups: upstream or initiator caspases, which include caspase-2, -8, -9, and -10, and downstream or effector caspases, which include caspase-3, -6, and -7.[7] The initiator caspases function as upstream signal transducers and trigger a cascade of downstream caspase activation; the effector caspases are responsible for the proteolytic cleavage of a broad range of cellular proteins, leading ultimately to cell death.[8] After animal debleeding, all skeletal muscle cells will irreversibly be deprived of oxygen and nutrients, and under these extremely harmful environmental conditions, they will have no alternative but to rapidly engage in cell death.[6,9] Some recent studies have proved apoptosis initiation in PM skeletal muscle cells by observing a rapid phosphatidylserine (PS) externalization, terminal deoxynucleotidyl transferase-mediated deoxyuridine triphosphate nick end-labeling (TUNEL) positive nuclei, condensation and margination of nuclear chromatin, and a progressive degradation of actin, which are considered as the typical features of apoptosis.[10−12] In addition, it was demonstrated that caspases were activated during PM storage of pork. These results, to some extent, provide prerequisites for further study on the contribution of caspases to PM proteolysis or tenderization.

At present, some of the compelling evidence indicating that the caspase system is involved in the PM tenderization of meat includes the following findings. A specific inhibitor of caspase-3 could attenuate the rate of PM tenderization or degradation of key myofibrillar proteins.[13] Changes in caspase activity[14] or expression of some factors[15] involved in apoptosis were significantly correlated with PM tenderness. Laville[16] found a higher quantity of proteins of the inner and outer membranes of mitochondria in tender meat by proteome analysis and suggested that it may be associated with a cell apoptotic process. Kemp[17] revealed that incubation of myofibrillar proteins from porcine skeletal muscle with caspase-3 resulted in the appearance of degradation products with molecular masses of 28 and 32 kDa and the degradation of desmin. However, Underwood[18] showed that caspase-3 activity is not activated and is also not associated with Warner−Bratzler shear force and, therefore, believed that caspase-3 is not likely involved in the PM tenderization of beef. The caspase system became a focus of attention in the field of meat aging since Herrera-Mendez[6] proposed that the caspase family is likely to be a candidate for PM tenderization. However, of the currently known, the direct data on the role of caspase in the conversion of muscle to meat are very limited.

An in vitro experimental study permits an investigation of a few or a single component with the exclusion of numerous other factors in the system and therefore is extensively employed in muscle biochemistry. It has been proposed and generally accepted that a protease system must reproduce PM changes in myofibrils in vitro under optimum conditions to be considered as a possible candidate to PM tenderization. Therefore, the objective of

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this study was to investigate in vitro proteolysis of myofibrillar proteins in bovine skeletal muscle by caspase-3 or -6 and whether the degradation pattern is similar to that observed in naturally aged beef. The reason for selecting the two caspases is that if the caspase system is involved in PM proteolysis of some cytoskeletal proteins, effector caspases are more likely to be direct participants. Among three effector caspases, caspase-3 and -7 share the same specific recognization sequence "DETD", while the specific recognization sequence for caspase-6 is "(L/E) EXD".[19]

## MATERIALS AND METHODS

**Animals and Muscle Sampling.** Three 2.5 years old crossbred cattle (Luxi × Simmental bulls with live weight 450 ± 50 kg) were slaughtered at a commercial meat processing company (HanSen Meat Co. Ltd., Anhui, China) according to the requirements of National Standards of P. R. China "Operating Procedures of Cattle Slaughter". After animal exsanguination, Longissimus thoracis (LT) muscles were excised from the right side of carcasses within 30 min and were frozen rapidly in liquid nitrogen for subsequent analysis.

**Preparation of Myofibrils.** Myofibrils were extracted according to the procedure as previously described[20,21] with small modification. Briefly, samples of muscle frozen in liquid nitrogen were allowed to thaw at room temperature. The samples were trimmed of visible fat and connective tissue and then cut into dices measuring approximately 1 $cm^3$. The minced muscle was homogenized in approximately 7.5 volumes of pyrophosphate relaxing buffer (PRB: 100 mM KCl, 2 mM $MgCl_2$, 2 mM EGTA, 1 mM DTT, 1 mM $NaN_3$, 2 mM $Na_4P_2O_7$, and 10 mM Tris-Maleate, pH 6.8) for two 30 s bursts at a speed of 14000 rpm using a Polytron (IKA, Germany), with a 30 s interval cooling period in between, and then, the homogenate was centrifuged at 1000*g* for 10 min. After the supernatant fluid was decanted, the pellet was resuspended in 10 volumes of low salt buffer (LSB: same as PRB except for pyrophosphate being omitted) and centrifuged as before. After four washings, the resultant pellet was resuspended in 10 volumes of Triton X-100 buffer (LSB supplemented with 0.5% w/v Triton X-100). The samples were then washed a further four times with LSB followed by two washes with 5 mM Tris-HCl buffer (pH 8.0) as above except that resuspension of the pellet was accomplished by vigorous stirring rather than by homogenization using a blender.

**Incubation Conditions.** Prior to incubation reactions, isolated myofibrils were washed two times with incubation buffer [IB: 50 mM HEPES, 10 mM DTT, 10% sucrose, 0.1% CHAPS, and 5 mM ethylenediaminetetraacetic acid (EDTA), pH 7.2]. The resultant pellet was resuspended in IB. The protein concentration was determined by the Bradford assay (Bio-Rad). A part of myofibrils were removed to serve as a time 0 sample before incubation. To investigate the influence of IB on in vitro degradation of myofibrillar proteins by proteases, we loaded samples without a wash with IB (0′) on every gel to compare with samples washed with IB (0).

Aliquots of 600 $\mu$g of isolated myofibrils were incubated in IB (designated as control) or the buffer containing 15 units of recombinant caspase-3 (Bioversion, CA) or 15 units of recombinant caspase-6 (Bioversion), respectively, where 1 unit is defined as the amount of enzyme that cleaves 1 nmol of DEVD-pNA for caspase-3 and 1 nmol of VEID-pNA for caspase-6, respectively, per hour at 37 °C. After 2 or 16 h of incubation at 30 °C, samples were centrifuged at 3000*g* for 5 min at 4 °C. The resultant pellet was taken for determination of protein concentration using the Bradford assay (Bio-Rad) and then mixed with treatment buffer [3 mM EDTA, 3% w/v sodium dodecyl sulfate (SDS), 20% v/v glycerol, 8% v/v $\beta$-mercaptoethanol, 0.04% w/v bromophenol blue, and 30 mM Tris-HCl, pH 8.0] to prepare samples for SDS−polyacrylamide gel electrophoresis (PAGE) and Western blotting. Subsequently, the samples were heated in a 50 °C water bath for 20 min and then centrifuged for 20 min (16000*g*). The resultant supernatant fluid was stored at −80 °C until loading.

**SDS-PAGE and Western Blotting.** The acrylamide percentage varied depending on the protein of interest: For titin and nebulin, 5% gels were used; for desmin, 10% gels were used; and for troponin-T and actin, 12.5% gels were used. All gels, except the 5% gels, included 4.5% stacking gels. The composition of the gels was as described in our previous paper.[13] The gels were electrophoresed on the Bio-Rad Mini-Protean II system (Bio-Rad Laboratories, Hercules, CA) at a constant voltage of 120 V for 10% and 12.5% gels and at a constant current setting of 4 mA/gel for 5% gel, respectively.

After electrophoresis, 5% gels were stained using Coomassie brilliant blue R-250, and the protein of interest in other gels was transferred onto polyvinylidine fluoride membranes (Millipore) in transfer buffer containing 25 mM Tris, 192 mM glycine, and 15% v/v methanol using a wet transfer apparatus (BioRad Laboratories). Membranes were blocked for 1 h at room temperature with 5% w/v nonfat dry milk in Tris-buffered saline containing Tween (TTBS: 0.05% Tween 20, 20 mM Tris, 137 mM NaCl, and 5 mM KCl). After blocking, the membranes were exposed to the following primary antibodies diluted in 5% w/v nonfat dry milk in TTBS for 16 h: mouse monoclonal antitroponin-T clone JLT-12 (Sigma), rabbit antiactin C-11 polyclonal antibody (pAb) (Sigma), and mouse monoclonal antidesmin clone DE-U-10 (Sigma). Subsequently, blots were washed with TTBS before exposed to the reciprocal secondary antibody. Finally, blots were again washed, and protein bands were detected using a Sigma fast DAB (3,3′-diaminobenzidine tetrahydrochloride) with metal enhancer tablet sets (Sigma). The gels or membranes were scanned with scanner (GT-800F EPSON) at a resolution of 600 dpi, and then, the densities of targeted bands were analyzed by Quantity One software (Bio-Rad).

**Statistical Analyses.** The data from three replicates (two gels/replicate) were analyzed by one-way analysis of variance using SAS statistical software (Version 8.2, SAS Inst.), and differences among mean values were determined by the least significant difference comparison procedure with a significance level of $P < 0.05$.

## RESULTS AND DISCUSSION

No detectable differences appeared between 0′ (without a wash with IB) and 0 (with a wash with IB) on all SDS-PAGE gels or Western blotting patterns, which demonstrated that IB had no or little influence on the degradation of myofibrillar proteins. The degradation patterns of proteins of interest were analyzed respectively as below.

**Degradation of Titin.** Titin is the largest single-chain protein identified to date, with a molecular mass of about 3000 kDa. The titin filament extends along the entire sarcomere from the Z-disk to the M-line, meanwhile it could interact with several other muscle proteins (actin, α-actinin, myosin, and so on); thus, it is considered to be a molecular ruler and plays an important role in sarcomere assembly.[22] It is, therefore, thought that PM degradation of titin could improve meat tenderness.[1,23] The intact form of titin is generally referred to as $T_1$, which is very easily degraded during PM aging.[24] In at-death muscle samples, titin occurred primarily as the $T_1$ form, and with aging time extended, $T_1$ was gradually degraded into $T_2$ form, which migrated only slightly faster under SDS-PAGE conditions than $T_1$. Meanwhile, a band with a molecular mass of ca. 1200 kDa also appeared with aging time extended, which has been identified as degradation fragment of titin in previous reports.[25−27] In our present study, no differences appeared between controls and time 0 samples after 2 h of incubation. However, as compared with control, incubation of myofibrillar proteins with both caspase-3 and -6 resulted in the

degradation of $T_1$ (Figure 1). Myofibrillar proteins incubated with caspase-3 for 2 h induced disappearance of $T_1$ band, and the density of the $T_2$ band increased significantly ($P < 0.05$) (Table 1). However, myofibrillar proteins digested with caspase-6 still possessed some $T_1$ through the same incubation time. Moreover, a band that migrated at approximately 1200 kDa was also present on 5% gels in both samples digested with caspase-3 and -6 (Figure 1), which was similar to that observed during normal PM aging.[26] To examine the effect of incubation time, we extended the incubation time over 12 h. In comparison with time 0 samples, $T_1$ in control samples was partially degraded after 12 h of incubation; the staining intensity of this band was approximately 43% of $T_1$ in the time 0 sample (Table 1). The degradation of $T_1$ in control samples possibly resulted from myofibril-bound proteolytic enzymes.[28−30] Wang[21] has also showed that myofibril-bound protease activities were difficult to be fully removed by washings. These factors may synergistically induce the degradation of $T_1$ after 12 h of incubation even without the addition of exogenous enzyme. After 12 h of incubation, no $T_1$ was noted in caspase-3-digested myofibrils and myofibrillar proteins incubated with caspase-6 only possessed approximately 5% of $T_1$. In comparison with control, the degradation of $T_1$ resulted from caspase-3 and -6 was significant ($P < 0.05$) (Table 1). As compared with 2 h of incubation, the relative value of $T_2$ from myofibrillar proteins incubated with caspase-3 for 12 h decreased slightly, which was likely to be caused by further degradation of $T_2$ by caspase-3. It has been demonstrated that the resultant $T_2$ polypeptide can be subsequently degraded or altered.[2,26] Overall, in vitro incubation of myofibrillar proteins from beef with caspase-3 and -6 could result in degradation of $T_1$ into $T_2$ fragments, with the appearance of 1200 kDa bands, which is in contrast to the findings reported by Mohrhauser,[31] who did not detect the degradation of $T_1$ after incubation of myofibrillar proteins with caspase-3 and is in line with the results in the naturally aged beef. Therefore, our present findings demonstrated that caspase-3 and -6 could in vitro mimic the degradation pattern of titin during normal PM aging of beef.

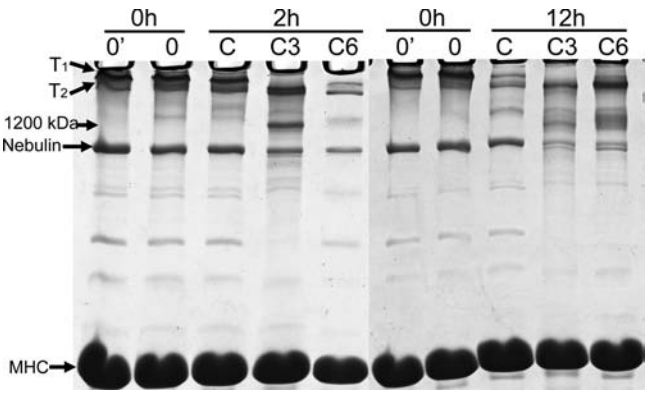


**Figure 1.** Representative Coomassie-stained 5% polyacrylamide gel illustrating titin and nebulin degradation after incubation of myofibrillar proteins with caspase-3 or -6 for 2 or 12 h at 30 °C. 0′ and 0 represent samples before or after dissolved in IB, respectively. C, control; C3, caspase-3; C6, caspase-6; $T_1$, intact titin; $T_2$, large degradation product of intact titin; and MHC, myosin heavy chain. Sixty micrograms of proteins was loaded per lane.

**Degradation of Nebulin.** Nebulin is another large molecular mass protein (600−900 kDa), which plays an important structural role in the force generating machinery of the muscle sarcomere by binding to the actin thin filament and regulating its assembly.[32] In the present study, degradation of nebulin in time 0 and control samples throughout the 12 h of incubation was not apparent, which differed from titin degradation. In comparison with control, incubation of myofibrillar proteins with caspase-3 and -6 resulted in significant degradation of nebulin ($P < 0.05$), and the levels of intact nebulin in both treatment groups were almost equivalent at the same incubation time, which left more than 40% at 2 h and decreased to less than 10% at 12 h (Table 1). In addition, a band, which migrated only slightly faster than intact nebulin, also appeared in samples treated with caspase-3 and -6, whereas there was no corresponding band appearing in control samples (Figure 1). Meanwhile, it has been documented that nebulin also migrates as a doublet after 1 day of PM storage in electrical stimulation (ES) samples[33] and at 3 days in no ES samples.[34] Therefore, our present results demonstrated that caspase-3 and -6 could in vitro reproduce the degradation pattern of nebulin during normal PM aging.

**28 and 32 kDa Fragments.** Although Troponin-T is not considered to be an important structure-maintaining protein, two fragments of approximately 28−32 kDa, which have been identified as degradation products from troponin-T by using Western blotting techniques, were shown to be an indicator of meat tenderization, due to their close relationship to meat

**Table 1. Relative Values of Targeted Myofibrillar Proteins of Bovine Skeletal Muscle Digested with Caspase-3 or -6 at 30 °C for 2 or 12 h as Compared with Undigested Controls[a]**

| | relative value of different targeted proteins | | | | | |
|---|---|---|---|---|---|---|
| | 2 h of incubation | | | 12 h of incubation | | |
| targeted proteins | C | C3 | C6 | C | C3 | C6 |
| $T_1$ | 0.98 ± 0.030 a | 0 c | 0.33 ± 0.020 b | 0.43 ± 0.021 e | 0 g | 0.05 ± 0.011 f |
| $T_2$ | 1.42 ± 0.041 c | 5.32 ± 0.108 a | 2.62 ± 0.076 b | 3.10 ± 0.058 g | 4.34 ± 0.076 f | 6.93 ± 0.068 e |
| nebulin | 0.98 ± 0.082 a | 0.44 ± 0.021 b | 0.42 ± 0.010 b | 0.80 ± 0.012 e | 0.09 ± 0.003 f | 0.09 ± 0.004 f |
| troponin-T | 0.99 ± 0.034 a | 1.04 ± 0.024 a | 1.07 ± 0.025 a | 1.18 ± 0.127 e | 1.16 ± 0.125 e | 1.11 ± 0.046 e |
| actin | 1.12 ± 0.033 a | 1.07 ± 0.098 a | 1.13 ± 0.052 a | 1.07 ± 0.013 e | 1.10 ± 0.011 e | 1.13 ± 0.046 e |
| desmin | 1.05 ± 0.048 a | 1.07 ± 0.023 a | 0.63 ± 0.011 b | 1.22 ± 0.046 e | 1.15 ± 0.065 e | 0 f |

[a] The relative value of targeted proteins was calculated as the density of targeted protein in different incubation conditions over the density of the targeted proteins in time 0 sample and was shown as the mean ± SD ($n = 3$). Letters a−c: means with different letters in a row of target proteins after 2 h of incubation differ at the 95% level. Letters e−g: means with different letters in a row of target proteins after 12 h of incubation differ at the 95% level. C, control; C3, caspase-3; C6, caspase-6; $T_1$, intact titin; and $T_2$, large degradation product of intact titin.

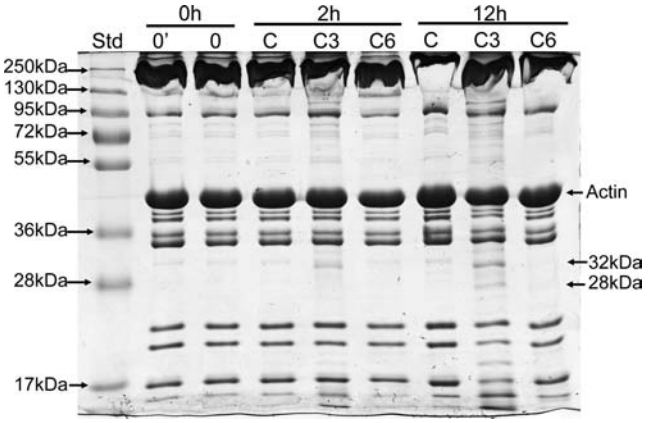


**Figure 2.** Representative Coomassie-stained 12.5% polyacrylamide gel illustrating the degradation of myofibrillar proteins after exposure to caspase-3 or -6 for 2 or 12 h at 30 °C. 0′ and 0 represent samples before or after dissolved in IB, respectively. C, control; C3, caspase-3; and C6, caspase-6. Twenty micrograms of proteins was loaded per lane.

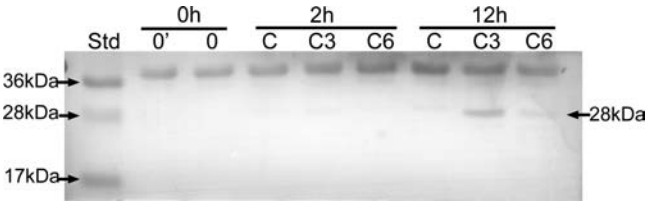


**Figure 3.** Representative Western blots of troponin-T in myofibrillar proteins after exposure to caspase-3 or -6 for 2 or 12 h at 30 °C. 0′ and 0 represent samples before or after dissolved in IB, respectively. C, control; C3, caspase-3; and C6, caspase-6. Twenty micrograms of proteins was loaded per lane.

tenderness.[24,26] In our study, we examined whether 28−32 kDa polypeptides occurred on 12.5% SDS-PAGE gels after caspase treatment.

In contrast to control, myofibrillar proteins incubated with caspase-3 for 2 h induced appearance of a 32 kDa polypeptide band, and the level of the polypeptide increased as the incubation time was extended (12 h). Furthermore, a band migrating at approximately 28 kDa also appeared at 12 h (Figure 2). The results were similar to that observed from normal PM aging. Meanwhile, our findings were also in accordance with the report by Kemp,[17] who found that bands with a molecular mass of 32 or 28 kDa appeared after incubation of porcine myofibrils with recombinant caspase-3. However, Mohrhauser[31] found no appearance of 28 or 32 kDa after digestion of myofibrils with caspase-3. To verify the origin of the 32 and 28 kDa fragments, we further performed a Western blotting experiment with a monoclonal antitroponin-T antibody. Results showed that as compared to control, although troponin-T in samples treated with caspase-3 has no significant degradation ($P > 0.5$) (Table 1), an immunoreactive band located at 28 kDa occurred on Western blotting pattern (Figure 3), which demonstrated that the 28 kDa polypeptide appeared on 12.5% Coomassie-stained gel was the degradation fragment of troponin-T by caspase-3. However, no immunoreactive band with the molecular mass of 32 kDa was labeled with monoclonal antitroponin-T antibody. Two possible mechanisms could be proposed from these results: One is that the 32 kDa polypeptide is derived from troponin-T, but the single

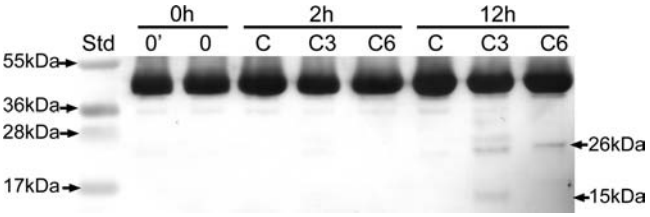


**Figure 4.** Representative Western blots of myofibrillar proteins labeled with antiactin antibody that recognized the C-terminal region after incubation with caspase-3 or -6 for 2 or 12 h at 30 °C. 0′ and 0 represent samples before or after dissolved in IB, respectively. C, control; C3, caspase-3; and C6, caspase-6. Twenty micrograms of proteins was loaded per lane.

epitope, which is detected by a monoclonal antitroponin-T, is not on the portion the of 32 kDa polypeptide and so the polypeptide was not recognized by the antibody; another is that the 32 kDa polypeptide was degradation fragment from other proteins but not troponin-T.

Numerous previous reports have demonstrated that caspase cleavage of purified actin could produce approximately 32 kDa fragment.[35] So, we examined whether the 32 kDa fragment occurred on 12.5% gels could be labeled with antiactin antibody that recognize the C-terminal region. As shown in Figure 4, two degradation fragments located at approximately 26 and 15 kDa, respectively, were apparently detected, but there was no visual immunoreactive band that occurred in the 32 kDa region. However, Kemp,[17] using MALDI-TOF mass spectrometry, has identified a 32 kDa fragment in caspase-3-digested myofibrils resulting from the proteolysis of actin. From our results and the report,[17] two different possibilities of the origin of 32 kDa fragment were given. One is that 32 kDa fragment appeared on 12.5% gels derives from other muscle proteins. Another is that the fragment indeed occur from the proteolysis of actin; however, it is not C-terminal region polypeptide of actin, which resulted in the unrecognizability by the antibody used in our study. Similar results to our present study have been reported by Communal,[35] who found that in vitro incubation of purified α-actin with caspase-3 produced three fragments (29, 20, and 15 kDa) on Coomassie-stained gels; however, the 30 kDa polypeptide was not detected using Western blotting approach. Although caspase caused actin proteolysis in vitro, the degradation pattern differed with the exhaustive degradation of actin resulted from lysosomal and proteasomal systems. As compared to control, there was no significant degradation of intact actin in caspase-3-digested myofibrils ($P > 0.5$) (Table 1). Furthermore, some groups have detected a degradation of actin during PM storage of muscle.[10,36,37] Therefore, the potential role of caspase-3 in PM tenderization should not be excluded like lysosomal and proteasomal systems.

Unlike the results with caspase-3 treatment, myofibrillar proteins digested by caspase-6 did not result in the appearance of bands at molecular mass of 28−32 kDa on Coomassie-stained gels (Figure 2). However, a 28 kDa band was labeled with monoclonal antitroponin-T antibody in caspase-6-digested samples (Figure 3), but the band was not as apparent as that in caspase-3-digested samples (Figures 2 and 3). The degradation pattern of actin was also different between samples treated with caspase-6 and -3. Only 26 kDa fragment was labeled with antiactin antibody in caspase-6-digested myofibrils (Figure 4). Therefore, caspase-3 and -6 only reproduced the 28 kDa fragment

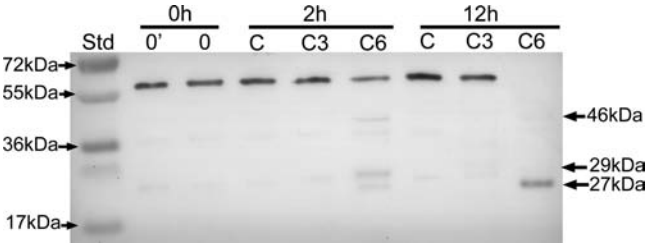


**Figure 5.** Representative Western blots of desmin in myofibrillar proteins after exposure to caspase-3 or -6 for 2 or 12 h at 30 °C. 0′ and 0 represent samples before or after dissolved in IB, respectively. C, control; C3, caspase-3; and C6, caspase-6. Twenty micrograms of proteins was loaded per lane.

derived from troponin-T, which occurred during PM storage of meat in vitro.

**Degradation of Desmin.** Desmin is the major muscle-specific intermediate filament protein. It plays an essential role in maintaining muscle cytoarchitecture by surrounding each Z-line and individual myofibrils interconnecting to one another and to the cell membrane at the level of their Z-discs.[38] It has been documented that desmin, together with titin, are likely key substrates that determine meat tenderness from a structural perspective.[1] In our present study, only minor intact desmin degradation was observed when myofibrils were digested with caspase-3 for 2 or 12 h (Figure 5). The results were inconsistent with the report by Kemp,[17] who found apparent degradation of desmin after incubation of myofibrillar proteins from pig with caspase-3. However, using a similar approach, Mohrhauser[31] has found no degradation of desmin. Incubation of myofibrillar proteins with caspase-6 resulted in significant degradation of intact desmin ($P < 0.05$) (Table 1). After 2 h of incubation, approximately 40% of intact desmin was cleaved by caspase-6; meanwhile, proteolytic products at 45, 29, and 27 kDa were also labeled by monoclonal antidesmin (Figure 5). After 12 h of incubation, intact desmin disappeared completely in caspase-6-digested samples; meanwhile, 45 and 29 kDa fragments were also invisible. Our results were in accordance with previous findings that desmin could be proteolyzed specifically in vitro by caspase-6 into 29 and 27 kDa products and none of the other effector caspases tested cleaved desmin.[39] During normal PM aging conditions, intact desmin is known to be gradually degraded, which can be also presented by caspase-6-digested myofibrils. Moreover, three degradation polypeptides (with molecular masses of 45, 38, and 35 kDa, respectively) from intact desmin have been reported to be occurred successively in the naturally aged beef.[26] In our study, incubation of myofibrillar proteins with caspase-6 resulted in the appearance of 45 kDa fragment. The 45 kDa fragment happens to be the degradation product from desmin that could not be produced by $\mu$-calpain-digested myofibrils.[26] Therefore, the degradation of desmin during PM storage possibly results from synergistic action between caspase and calpain. It has been reported that direct cleavage by calpain can lead to inactivation of caspase.[40] So, effector caspases maybe function in proteolysis of muscle proteins before calpain activation.

Overall, the study demonstrated that recombinant caspase-3 or -6, in vitro, could result in proteolysis of beef myofibrillar proteins including titin, nebulin, troponin-T, actin, and desmin. The degradation patterns of titin and nebulin were similar to those observed during normal PM aging. Both caspases only reproduced 28 kDa derived from troponin-T occurred during PM storage of beef. Caspase-6 induced increasing degradation of intact desmin with incubation time extended, but caspase-3 caused little cleavage of intact desmin. Among the three degradation fragments (45, 29, and 27 kDa) from intact desmin by caspase-6, only the 45 kDa fragment was observed in naturally aged beef, which could not be produced in $\mu$-calpain-digested myofibrils. Therefore, caspase-3 or -6 could only reproduce a part of myofibrillar protein degradation or degradation fragments observed in naturally aged muscle and are likely involved in PM proteolysis of muscle proteins together with other endogenous proteolytic enzymes.

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