INHIBITION OF POSTMORTEM TENDERIZATION IN OVINE CARCASSES THROUGH INFUSION OF ZINC

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ABSTRACT

Ovine carcasses were infused with a volume equal to 10% of their live weight with 50 m*M* $ZnCl_2$. Infusion of carcasses with $ZnCl_2$ blocked the postmortem tenderization process. Between d 1 and d 14, no change occurred in the shear force values or myofibrillar fragmentation index of chops obtained from $ZnCl_2$ -infused carcasses. Infusion of $ZnCl_2$ also prevented the proteolysis of myofibrillar proteins that occurs during postmortem aging. Infusion of $ZnCl_2$ has no effect on the activities of cathepsins B and B+L measured at 1 and 14 d postmortem. Although $ZnCl_2$ infusion prevented the loss of Ca^{2+}-dependent protease inhibitor activity that occurs during postmortem storage, it had no effect on the Ca^{2+}-dependent proteases. Studies in vitro indicated that $ZnCl_2$ inhibited the activities of Ca^{2+}-dependent proteases and cathepsins B and B+L. Hence proteolysis of myofibrillar proteins must play a key role in postmortem tenderization. Because zinc inhibited activities of both the Ca^{2+}-dependent proteases and the cathepsins, the relative contributions of these two classes of proteases cannot be assessed. Other proteases also could be involved in this process; however, their activity must be regulated by Ca^{2+} and Zn^{2+}.
(Key Words: Tenderness, Postmortem Changes, Proteolysis, Zinc, Ca^{2+}-dependent Proteases, Lysosomes.)

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Introduction

We have known for almost a century that meat tenderness increases gradually as a result of postmortem storage of carcasses at refrigerated temperatures (Lehmann, 1907). Although this improvement in meat tenderness is measurable both subjectively and objectively, the mechanism responsible for this change remains controversial. Hoagland et al. (1917) reported that proteolysis plays an important role in this process. Since then, much evidence in support of proteolysis has been reported (Penny, 1980; Goll et al., 1983; Dutson 1983; Koohmaraie, 1988). Because proteolytic changes in collagen during postmortem storage comparable to those of myofibrillar proteins have not been observed, the principal mechanism of meat tenderization during postmortem storage may be limited to hydrolysis of myofibrillar proteins (Tarrant, 1987). However, in spite of overwhelming evidence in support of proteolysis, some believe that proteolysis has no role in this process and that these changes are induced nonenzymatically by Ca^{2+} (Takahashi et al., 1987).

Whiting and Richards (1978) reported that injecting pre-rigor muscle with zinc increased shear force of the meat after aging. In addition, the zinc concentration of muscle, in combination with three other ions, also has been correlated positively with shear force (Vavak et al., 1976). Seideman et al. (1984) also reported that Zn content may be related to the tenderness of meat. However, Field et al. (1985) did not find a relationship between zinc concentration and meat tenderness. The results

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of experiments conducted in this laboratory have indicated that Ca^{2+}-dependent proteases (**CDP**) probably play a key role in the postmortem tenderization process (Koohmaraie, 1988). Because zinc inhibits the activity of CDP (Guroff, 1964), the objectives of these experiments were to examine the effect of zinc on the postmortem tenderization process.

Materials and Methods

Experiments In Vivo. A total of 12 lambs (1/4 Suffolk × 1/4 Finnsheep × 1/8 Dorset × 1/8 Rambouillet, 5 to 7 mo old, 35 to 45 kg live weight) were slaughtered on the same day. Six lambs were assigned as controls and the other six for $ZnCl_2$ infusion.

Infusion and Sampling. After bleeding (through only one jugular vein) the lamb carcasses were transferred to a lamb cradle and the carotid artery was exteriorized. One jugular vein remained intact; the other was opened and the carotid artery not used for infusion was clamped to prevent backflow. Zinc chloride (50 m*M*) then was infused (10% of the live weight) into the artery. After completion of the infusion, the carcasses were dressed and transferred to a holding cooler (1 to 2°C). Twenty-four hours after slaughter, the entire loin was removed, divided into two sections and assigned alternately to d 1 or d 14 postmortem for the following determinations: shear force, zinc content, myofibrillar fragmentation index, activities of Ca^2-dependent and lysosomal proteases and sodium-dodecyl sulfate polyacrylamide gel electrophoresis (**SDS-PAGE**) of isolated myofibrils at d 1. The same measurements except for zinc content analysis were taken at d 14.

Preparation of Ca^{2+}-Dependent Proteases and Their Inhibitor. Low and high calcium-requiring forms of Ca^{2+}-dependent proteases (CDP-I and CDP-II, respectively) and their inhibitor (CDP inhibitor) were prepared and assayed from 100 g of longissimus muscle after 1 and 14 d of postmortem storage. Quantification of CDP and their inhibitor was done according to the procedure described by Koohmaraie (1990), except that dialysis of muscle extract, instead of addition of cold deionized water, was used to reduce ionic strength prior to ion-exchange chromatography.

Preparation of Lysosomal Proteases. Lysosomal proteases were prepared after 1 and 14 d postmortem according to the procedure of Bechet et al. (1986). Briefly, the samples were homogenized (Polytron, 3 × 15 s, 1/2 speed) in 7 volumes of ice-cold .25 *M* sucrose, pH 7.2, containing .15 *M* KCl and 1 m*M* EDTA and centrifuged at 1,000 × g_{max} for 10 min and then 4000 × g_{max} for another 20 min. The supernatant was passed through glass wool and then centrifuged at 25,000 × g_{max} for 20 min to collect the lysosomal fraction. The lysosomal-enriched fraction then was lysed with 50 m*M* sodium acetate buffer, pH 5.0, containing 1 m*M* EDTA and .2% Triton X-100 and frozen at –20°C for at least 24 h. After thawing, the lysate was clarified by centrifugation at 32,000 × g_{max} for 40 min. After determination of protein content, the activity was determined fluorimetrically according to Kirschke et al. (1983). Cathepsin B was assayed with N-CBZ-L-arginyl-L-arginine 7-amido-4 methylcoumarin (**Z-Arg-Arg-NHMec**) as substrate and cathepsins B+L were assayed together with their common substrate N-CBZ-L-phenylalanyl-L-arginine 7-amido-4 methylcoumarin (**Z-Phe-Arg-NHMec**).

Myofibril Fragmentation Index. The myofibril fragmentation index (**MFI**) was determined according to the procedure described by Culler et al. (1978).

Myofibrillar Isolation. Myofibrils were isolated according to the procedure described by Olson et al. (1976).

Sodium-Dodecyl Sulfate Polyacrylamide Gel Electrophoresis. SDS-PAGE was performed on the isolated myofibrils according to Laemmli (1970) with 7.5 to 15% gradient gels (acrylamide:bisacrylamide, 75:1). The acrylamide solution contained 50% glycerol. Fifteen percent sucrose was included in the 15% separating gel.

Shear Force. Shear force of cooked chops (two chops per animal at each postmortem time) was determined according to the procedure outlined by AMSA (1978). Briefly, chops were cooked to an internal temperature of 70°C on Farberware Open Hearth broilers. The internal temperature of each chop was monitored by iron/constantan thermocouples placed in the geometric center of each chop. Chops were cooled overnight (4°C). Three cores (1.27 cm diameter) were removed from each chop parallel to the fiber direction. Each core was sheared with a Warner-Bratzler shear device attached to an Instron Universal Testing machine (Model 1132) with a microprocessor (Microcon II).

TABLE 1. LEAST SQUARE VALUES FOR SHEAR FORCE, ZINC CONTENT, MYOFIBRILLAR FRAGMENTATION INDEX (MFI) AND PROTEASE ACTIVITY FOR LONGISSIMUS MUSCLE FROM CONTROL AND $ZnCl_2$-INFUSED CARCASSES AT TWO DIFFERENT AGING PERIODS

	Control		$ZnCl_2$-infused			Probability level		
	d 1	d 14	d 1	d 14	SE	$ZnCl_2$ effect	Aging effect	Interaction
Zn, μg/g	8.25		62.48			.01		
Shear force[a]	11.47	5.72	11.25	10.13	.40	.01	.01	.01
MFI	43.63	75.92	39.07	39.67	2.20	.01	.01	.01
CDP-I[b]	76.93	17.26	115.13	22.78	4.54	.01	.01	.01
CDP-II[c]	115.19	109.30	126.01	92.88	6.79	.77	.02	.07
CDP inhibitor[d]	125.53	9.96	142.18	187.47	16.73	.01	.06	.01
Cathepsin b[e]	85.17	105.82	93.88	97.57	7.61	.98	.14	.29
Cathepsins B+L[e]	77.62	90.72	92.63	84.09	7.31	.71	.76	.17

[a]kg/1.27 cm core.

[b]Low-calcium-requiring Ca^{2+}-dependent protease. Total activity/100 g muscle (caseinolytic activity).

[c]High-calcium-requiring Ca^{2+}-dependent protease. Total activity/100 g muscle (caseinolytic activity).

[d]Total activity/100 g muscle (inhibition of casein hydrolysis by CDP-II).

[e]Pmol of product released · min^{-1} · g $muscle^{-1}$.

Zinc. Water extractable zinc content of the longissimus muscle was determined by atomic absorption according to the procedure described by Nakamura (1973a,b).

Statistical Analysis. Data were analyzed by least squares procedures (SAS, 1985). Treatments were considered a whole-plot effect and days postmortem and the interaction between days postmortem and treatment were considered a split-plot effect. Error term used to test treatment effect was the mean square of animal (treatment), whereas other effects were tested by the residual mean square.

Experiments In Vitro. Ca^{2+}-dependent proteases (CDP-I and CDP-II) were prepared from ovine skeletal muscle by ion-exchange chromatography. Chromatographic fractions containing CDP-I and CDP-II activities were pooled separately, salted-out between 0 and 45% ammonium sulfate, dialyzed and rechromatographed. However, the data reported here were generated from four different preparations of CDP-I and CDP-II that were obtained from ovine skeletal muscle and prepared according to Koohmaraie (1990) omitting salting-out procedures. A stock solution of 100 m*M* $ZnCl_2$ was prepared and diluted with Ca^{2+}-media (100 m*M* Tris, 5 m*M* $CaCl_2$, 5 mg/ml casein and 10 m*M* 2-mercaptoethanol, pH adjusted to 7.5 with 1*N* acetic acid) to give an appropriate concentration of $ZnCl_2$ (i.e., 0 to 1 m*M*). Lysosomal enzymes were prepared according to the procedure of Bechet et al. (1986) as described above but omitting EDTA from all solutions. Dilutions were made from 100 m*M* $ZnCl_2$ to give an appropriate concentration of $ZnCl_2$ (i.e., 0 to 1 m*M*) when assaying cathepsin B (activity against Z-Arg-Arg-NHMec) and cathepsins B+L (activity against Z-Phe-Arg-NHMec).

Results

Infusion of lamb carcasses with 50 m*M* $ZnCl_2$ immediately after death completely blocked the postmortem changes normally observed in the longissimus muscle (Table 1). That is, no changes occurred in shear force values or myofibril fragmentation index of chops obtained from the $ZnCl_2$-infused animals during postmortem storage between d 1 and d 14 (Table 1, Figure 1).

To examine the effect of $ZnCl_2$ infusion on the degree of fragmentation and integrity of Z-lines, myofibrils prepared from the same preparation used for determination of MFI after 14 d of postmortem storage were examined by phase microscopy (Figure 1). These results clearly substantiated those of MFI values obtained a d 14 (Table 1), in that myofibrils prepared from control samples were highly fragmented (Figure 1), whereas those infused with $ZnCl_2$ basically remained intact (Figure 1B).

To examine the effect of $ZnCl_2$ on postmortem proteolysis of myofibrillar proteins, myofibrils were prepared from control and $ZnCl_2$-

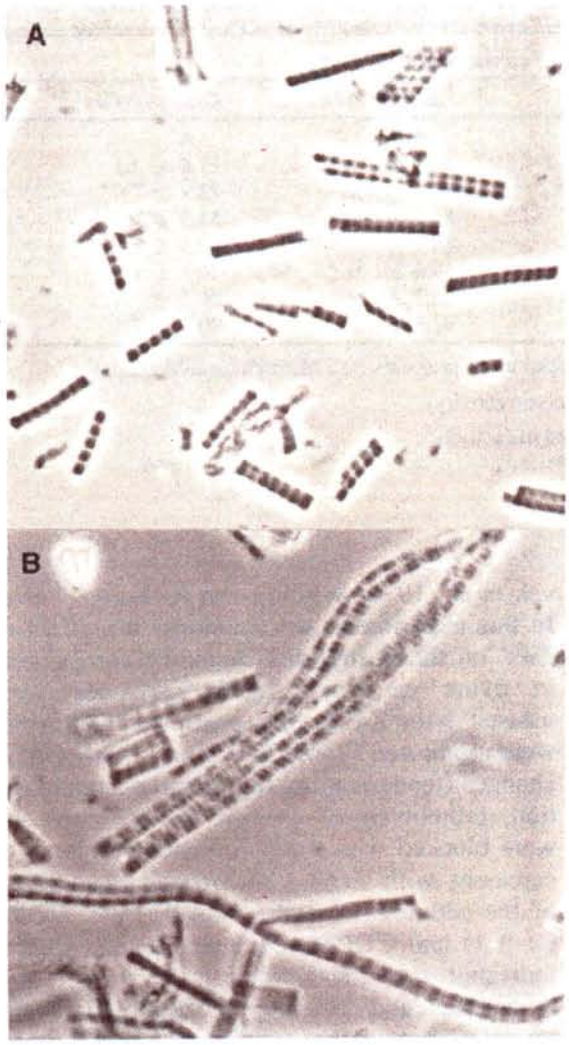

Figure 1. Phase micrograph of myofibrils prepared from Control (A) and $ZnCl_2$-infused (B) longissimus muscle after 14 d of postmortem storage Width of field in photomicrograph is 40 μm.

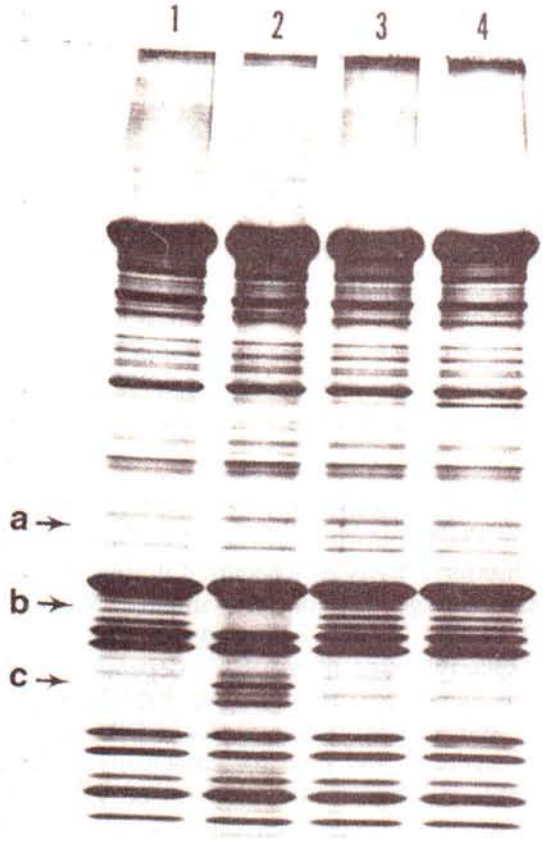

Figure 2. Polyacrylamide gel electrophoresis of myofibrillar proteins isolated from Control and $ZnCl_2$-infused animals after 1 and 14 d of postmortem storage. Seventy micrograms of myofibrillar proteins were electrophoresed under denaturing conditions on 7.5 to 15% gradient gels. 1 = Control, d 1; 2 = Control, d 14; 3 = $ZnCl_2$-infused, d 1; 4 = $ZnCl_2$-infused, d 14. Arrows a, b and c indicate positions of desmin, troponin-T and 30,000 dalton fragments, respectively.

infused longissimus muscle after 1 and 14 d of postmortem storage at 2°C and subjected to SDS-PAGE (Figure 2). In support of the previously mentioned results, no detectable proteolysis of myofibrillar protein occurred in the longissimus muscle of $ZnCl_2$-infused animals. In contrast, the following changes were observed in the control samples: 1) disappearance of troponin-T; 2) appearance of 30,000 dalton component; and 3) disappearance of desmin. Appearance of 30,000 dalton component has been reported to be the most consistent and noticeable change that occurs in myofibrillar proteins during postmortem storage (Goll et al., 1983; Koohmaraie, 1988).

To examine the effect of $ZnCl_2$ infusion on the classes of proteases thought to be involved in the postmortem tenderization process, the activities of CDP-I, CDP-II, CDP inhibitor, cathepsins B and B+L were measured after 1 and 14 d of postmortem storage (Table 1). Results indicated that $ZnCl_2$ infusion had no effect on the lysosomal enzymes and CDP-II. The differences in CDP-I activity between control and $ZnCl_2$-infused samples were significant at d 1 postmortem ($P < .01$) but not at d 14. However, the difference in CDP inhibitor between control and $ZnCl_2$-infused samples

TABLE 2. EFFECT OF Zn ON THE ACTIVITIES OF Ca^{2+}-DEPENDENT AND LYSOSOMAL PROTEASES

	Percent inhibition[a]			
Zinc μM	CDP-I[b]	CDP-II[c]	Cathepsin B[d]	Cathepsins B+L[e]
0	.0	.0	.0	.0
5	48.2 ± 1.4	50.5 ± 1.9	52.3 ± 1.9	17.6 ± 2.5
10	66.1 ± 1.9	69.6 ± 3.1	67.9 ± 1.9	23.1 ± 7.9
25	89.5 ± 1.4	92.0 ± 4.9	84.9 ± 1.4	51.5 ± 1.2
50	100.0	100.0	88.7 ± 1.0	69.3 ± 2.1
100	100.0	100.0	89.6 ± .9	79.1 ± 1.6
250	100.0	100.0	88.7 ± .9	86.5 ± .9
1000	100.0	100.0	92.3 ± 1.2	90.4 ± 2.1

[a]Mean ± SEM of four different ovine skeletal muscle preparations of proteases (see Materials and Methods).

[b]Low-Calcium-requiring Ca^{2+}-dependent protease (cascinolytic activity).

[c]High-Calcium-requiring Ca^{2+}-dependent protease (cascinolytic activity).

[d]Activity against Z-Arg-Arg-NHMec.

[e]Activity against Z-Phe-Arg-NHMec.

were significant at d 1 and d 14 postmortem. The $ZnCl_2$ infusion prevented the loss in CDP inhibitor activity that occurred during postmortem storage in control samples (Table 1).

Studies in vitro indicated that $ZnCl_2$ was a potent inhibitor of the Ca^{2+}-dependent proteases and cathepsins. The percent inhibition at 50 μM $ZnCl_2$ was 100 for CDP, 88.9 for cathepsin B and 69.3 for cathepsins B+L. To examine whether the binding of Zn^{2+} is a reversible reaction, the following experiments were conducted. For CDP, CDP-II was incubated with 100 μM $ZnCl_2$ and after 30 min the activity was determined. No activity could be detected. The CDP-II + Zn^{2+} was then dialyzed and rechromatographed according to the procedure described in Materials and Methods. Results indicated that more than 95% of the original activity was recovered (37.3 units of activity were loaded; 36.2 and 36.8 units were recovered from control and $ZnCl_2$-incubated samples, respectively). For lysosomal enzymes, the samples were incubated with 1 mM $ZnCl_2$ and after a 30-min incubation the EDTA concentration was increased from 0 to 4 mM and assayed for cathepsins B and B+L. No inhibition was observed. These results indicate that the Zn^{2+}-induced inhibition is reversible.

Discussion

As early as 1964 (Guroff, 1964), Zn^{2+} ions were known to be potent inhibitor of Ca^{2+}-dependent proteases. Also, there have been indications that Zn^{2+} has an inhibitory effect on the postmortem tenderization process (Vavak et al., 1976; Whiting and Richards, 1978). In this experiment, we examined the effect of Zn^{2+} on the postmortem tenderization process in ovine carcasses. Lamb carcasses were infused with $ZnCl_2$ and postmortem changes were monitored (Table 2). Clearly, postmortem changes (tenderization, myofibrillar fragmentation, proteolysis of specific muscle proteins) were blocked with $ZnCl_2$ infusion. Infusion of carcasses with $ZnCl_2$, however, had no effect of the activities of cathepsins B, B+L, CDP-I (at d 1) and CDP-II. Because in vitro studies indicated that the inhibition by $ZnCl_2$ was reversible, and because the extraction and measurement of the activities of these proteases (from $ZnCl_2$-infused carcasses) were done under conditions that would reverse the effect of $ZnCl_2$, it is rather difficult to make a statement regarding the activity of these proteases in $ZnCl_2$-infused carcasses.

Results of these experiments suggest strongly that proteolysis of myofibrillar proteins is a major reason for the observed improvement in meat tenderness during postmortem storage of carcasses. However, because zinc inhibited activities of both the Ca^{2+}-dependent proteases and the cathepsins, based on these results alone it cannot be concluded which class of proteases is involved in the meat tenderization process. However, the combination of these and other results suggests that perhaps zinc induces its effect (i.e., inhibition of postmortem tenderization) through inhibition of the activities of CDP. For example: 1) infusion of carcasses with $CaCl_2$ results in the acceleration of the postmortem tenderization process (Koohmaraie et al., 1988b, 1989); 2)

$CaCl_2$ at 10 m*M* inhibits the activity of cathepsin B by 39% (Barrett, 1973); 3) lysosomal enzymes cause hydrolysis of some myofibrillar proteins that does not occur during postmortem storage (e.g., myosin). The best evidence is the recent report by Bandman and Zdanis (1988), who failed to detect any myosin degradation for up to 4 wk of postmortem storage at 4°C. This supports SDS-PAGE studies by a large number of investigators (Arakawa et al., 1976; Olson et al., 1977; Penny, 1980; Koohmaraie et al., 1984a,b,c, 1986, 1988a). Finally, lysosomal enzymes normally are located in the lysosomes and presumably have to be released to have access to the substrate (e.g., myofibrils). It has been assumed that during postmortem storage lysosomes are ruptured and thereby cathepsins are released into cystosol. However, the only experiment conducted in this area has indicated the opposite: even after electrical stimulation and 30 d of carcass storage, lysosomal rupture was not apparent (LaCourt et al., 1986).

The effect of $ZnCl_2$ infusion of carcasses on the components of Ca^{2+} dependent proteolytic system is interesting. Previous studies have indicated that under normal postmortem conditions (i.e., slaughter and holding of carcasses at 2°C for up to 14 d) CDP-II is remarkably stable, whereas there is a gradual decline in activity of CDP-I, and CDP inhibitor loses its activity rapidly (Vidalenc et al., 1983; Ducastaing et al., 1985; Koohmaraie et al., 1987). Both CDP-I and CDP-II undergo autolysis in the presence of Ca^{2+} with the eventual loss of activity (Guroff, 1964; Suzuki et al., 1981a,b; Hathaway et al., 1982; Mellgren et al., 1982; Parkes et al., 1985; DeMartino et al., 1986; Imajoh et al., 1986; Inomata et al., 1986; Crawford et al., 1987). The loss of enzymatic activity due to autolysis is highly temperature-dependent (Koohmaraie et al., 1989) and greatly reduced in the present of substrate (DeMartino et al., 1986). We have suggested that autolysis is the reason for loss of CDP-I activity during postmortem storage at 2°C (Koohmaraie et al., 1987). Another explanation for loss of CDP-I activity could be its hydrolysis by another protease. Current data support the latter explanation because Zn^{2+} inhibited CDP-I activity (Table 2) and its activity continued to decline in $ZnCl_2$-infused samples (Table 2). Given these results, it would be unlikely that autolysis could explain the loss of CDP-I activity during postmortem storage. Previous work (Koohmaraie et al., 1987) and results reported here (Table 1) document that in control animals CDP-I retains about 50 to 60% of original activity after 24 h of postmortem storage. These values are derived from the following facts: the ratio of CDP-I to CDP-II in skeletal muscle is 1:1, and because CDP-II activity is unaffected by postmortem storage, CDP-II activity at 24 h can be used to estimate the initial activity of CDP-I and, consequently, the percentage of CDP-I activity remaining after 24 h. In contrast to the control animals, which retained 50 to 60% of CDP-I activity at 24 h, the $ZnCl_2$-infused samples retained about 100% of their original CDP-I activity. Therefore, it can be argued that due to inhibition of CDP-I by Zn^{2+}, no autolysis has occurred. The fact that $ZnCl_2$-infused animals exhibited a loss in CDP-I activity from d 1 to d 14 that paralleled the decrease in CDP-I activity in control animals suggests that the demonstrated decrease in CDP-I activity may result from proteolytic activity other than autolysis and cannot be prevented by Zn^{2+} inhibition of CDP-I activity. Further experimentation will be needed to determine the mechanism of loss of CDP-I activity during postmortem storage.

As mentioned previously, of the three components of Ca^{2+}-dependent proteolytic system, CDP inhibitor is the most susceptible (greatest loss of activity) to postmortem storage. Results of this experiment indicated that in samples from $ZnCl_2$-infused sheep, CDP inhibitor retained its activity. After 14 d of postmortem storage at 2°C, control samples had only about 8% of the CDP inhibitor present at d 1, whereas in samples from animals infused with $ZnCl_2$ more CDP inhibitor was observed than on d 1. Clearly, $ZnCl_2$ infusion prevented the loss of CDP inhibitor activity during postmortem storage. Reasons for the rapid loss of CDP inhibitor in samples obtained from control animals during postmortem storage are not apparent yet. However, both CDP-I and CDP-II are capable of hydrolyzing CDP inhibitor (Goll et al., 1985). Because Zn^{2+} inhibits the activities of both CDP-I and CDP-II (Table 1), and because $ZnCl_2$ infusion prevented the loss in the activity of CDP inhibitor (Table 2), it is tempting to speculate that the loss of CDP inhibitor activity could occur through hydrolysis by Ca^{2+}-dependent proteases. However,

further experimentation is required to test the accuracy of this speculation.

The results of this study clearly demonstrate that Zn^{2+} is a potent inhibitor of the Ca^{2+}-dependent proteases under in vitro conditions. Whether Zn^{2+} regulates the activity of these proteases in vivo remains to be determined. However, literature results suggest that Zn^{2+} may play a significant role in the postmortem tenderization process. Cassens et al. (1967) demonstrated that red skeletal muscles (for example, psoas major) had threefold to fourfold higher ~~Ca^{2+}~~ Zn^{2+} content than did white skeletal muscles (for example, longissimus). The largest portion of muscle zinc (64% and 86% in the white and red muscle, respectively) was found in the fraction composed primarily of myofibrils and nuclei. Olson et al. (1976) demonstrated that the myofibril fragmentation index of bovine longissimus and semitendinosus increased considerably from 1 to 6 d postmortem (interpreted as tenderization), whereas in the psoas major the MFI increased only slightly (interpreted as little or no postmortem change in tenderness). Koohmaraie et al. (1988c) reported that during a 14-d postmortem storage, shear force values decreased greatly for bovine longissimus (3.31 kg) but only slightly for psoas major (.16 kg).

The results of this study indicate that the quantification of Ca^{2+}-dependent proteases for comparative studies may be useful. However, the results should be interpreted with caution, because the levels of Ca^{2+}-dependent proteases recovered from skeletal muscle may not accurately reflect the activity of these proteases in vivo due to the concentrations of Zn^{2+} in the cellular compartments(s) containing these proteases and other regulators such as the CDP inhibitor, the purported CDP activator and others yet to be identified.

Implications

Proteolysis of myofibrillar proteins is a major reason for the postmortem tenderization of meat that occurs under refrigerated conditions. The Ca^{2+}-dependent proteases probably play a key role in the proteolysis of myofibrillar proteins during postmortem storage; protease(s) other than Ca^{2+}-dependent proteases could be involved. It would appear, however, that their activity must be stimulated by Ca^{2+}, inhibited by $ZnCl_2$ and be endogenous to skeletal muscle cells.

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