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Effect of Post-mortem Storage on Ca^{++}-Dependent Proteases, Their Inhibitor and Myofibril Fragmentation*

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SUMMARY

Post-mortem changes in two calcium-dependent proteases, their inhibitor, myofibril fragmentation index (MFI) and collagen (amount and solubility) were studied. Whereas the activity of high Ca^{++}-requiring calcium-dependent protease (CDP-II) remained nearly constant throughout post-mortem storage, there was a progressive decrease in the activities of low Ca^{++}-requiring calcium-dependent protease (CDP-I) and their specific inhibitor, with the inhibitor being the most susceptible to post-mortem storage. Results indicated that the greatest changes in MFI occur within the first 24 h of post-mortem storage. There were no detectable changes in either total or soluble collagen content with post-mortem storage. Hence, it was concluded that improvement in tenderness resulting from post-mortem storage must be derived from changes in the myofibrils and since CDP-I activities paralleled the myofibrillar changes, it seems reasonable to suggest that CDP-I, not CDP-II, plays an important role in the fragmentation of myofibrils and consequently in improvement of meat tenderness resulting from post-mortem storage.

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INTRODUCTION

Over the years, a large number of studies have demonstrated that post-mortem storage at 0 to 5°C dramatically improves meat tenderness (for review, see Jeremiah, 1978; Penny, 1980). Also, there has been considerable effort directed toward understanding the mechanism(s) responsible for this improvement in meat tenderness during post-mortem storage. However, the conclusions are unclear and the mechanism of post-mortem tenderization is still a controversial issue. It is generally accepted that the majority, if not all, of this improvement is the result of proteolytic degradation by proteases endogenous to the skeletal muscle cell (for review see Goll *et al.*, 1983). Among the proteases located inside the skeletal muscle cell, Ca^{++}-dependent protease(s) seem to be the best candidate(s) as a possible mechanism for proteolytic degradation of myofibrils during post-mortem storage (Goll *et al.*, 1983). The latest report regarding the effect of low Ca^{++}-requiring Ca^{++}-dependent protease (CDP-I) on the myofibrils under actual post-mortem conditions by Koohmariae *et al.* (1986) appears to strengthen the hypothesis that Ca^{++}-dependent protease may play a major role during post-mortem tenderization of meat.

A well documented change in the myofibril as a result of post-mortem storage is its susceptibility to fragmentation upon homogenization (Penny, 1980). This increased susceptibility to fragmentation upon homogenization is the result of the weakening of the Z-disks. Change in the degree of fragmentation, although a measure of structural alterations in the native myofibrils, correlates significantly with the increase in tenderness of meat cooked after post-mortem storage. Indeed, Moller *et al.* (1973) obtained a correlation of 0·78 between myofibril fragmentation and sensory tenderness of bovine *longissimus* muscle.

In view of the above consideration, these studies were conducted to examine the effect of post-mortem storage on the activities of the low Ca^{++}-requiring (CDP-I) and the high Ca^{++}-requiring (CDP-II) Ca^{++}-dependent proteases, their inhibitor, myofibril fragmentation index and connective tissue.

MATERIALS AND METHODS

Animals

Samples were obtained from *longissimus* muscle* from 15 A-maturity cattle with carcass weights ranging from 217 to 445 kg.

* The term *longissimus* refers to *M. longissimus dorsi.*

Preparation of Ca^{++}-dependent proteases and their inhibitor

Low Ca^{++}-requiring (CDP-I) and high Ca^{++}-requiring (CDP-II) Ca^{++}-dependent proteases and their inhibitor were isolated from 200 g of *longissimus* muscle immediately after slaughter and after 1, 6 and 14 days of post-mortem storage (1°C). The muscles were taken to a cold room (2°C), trimmed of fat and connective tissue, cut into 2 × 2 cm pieces and immediately frozen in liquid nitrogen and stored at −70°C. At the time of extraction, samples were removed from −70°C and allowed to stand at 2°C for 90 min. Samples were then homogenized in 2·5 volume of 10 mM Tris-HCl, pH 7·5, containing 4 mM EDTA, 50 mM NaCl and 2 mM 2-mercaptoethanol (MCE), in a Waring blender. Homogenization was done twice for 30 s at the low speed setting and twice for 30 s at high speed setting, with a 30-s cooling period interspersed between the bursts. After a 1 h extraction with gentle mixing, the homogenate was centrifuged at 30 000 × g_{max} for 50 min. The supernatant was filtered through two layers of cheese cloth and the pH of the supernatant was adjusted to 7·5 followed by centrifugation at 50 000 × g_{max} for 50 min. The supernatant of 50 000 × g_{max} was filtered through glass wool and chromatographed on DEAE-Sephacel that had been equilibrated with 5 mM Tris-HCl, pH 7·40, containing 50 mM NaCl, 0·1 mM EDTA, 2 mM MCE. The bound proteins were eluted with a continuous gradient of 50–500 mM NaCl in 5 mM Tris-HCl, pH 7·40, containing 50 mM NaCl, 0·1 mM EDTA, 2 mM MCE.

Measurements of activities

Activities of CDP-I were assayed according to Koohmaraie *et al.* (1986) and CDP-II according to Koohmaraie *et al.* (1984). Incubation times were 50 min. The inhibitor activity was determined by pre-incubating appropriate amounts of inhibitor and enzyme at 25°C for 1 min before adding 1·5 ml of reaction mixture to start the reaction. The reaction mixture consisted of 100 mM tris-acetate, pH 7·5, 10 mM MCE, 5 mg/ml casein and either 100 μM $CaCl_2$ (CDP-I) or 5 mM $CaCl_2$ (CDP-II). In the case of EDTA containing reaction mixture $CaCl_2$ was replaced by 10 mM EDTA. The reaction was subsequently stopped by adding an equal volume of 5% trichloroacetic acid. For each fraction the following measurements were made to determine inhibitor activity:

(a) Enzyme alone in Ca^{++}-containing reaction mixture (to determine Ca^{++}-dependent caseinolytic activity).
(b) Fraction being assayed alone in EDTA-containing reaction mixture (to determine Ca^{++}-independent activity).
(c) Enzyme + inhibitor in Ca^{++}-containing reaction mixture.

TABLE 1
Changes Associated with Post-mortem Storage (Mean ± SEM)*

	Days post mortem					
	0	*1/2*	*1*	*3*	*6*	*14*
Shear force (kg)	—	—	7·71 ± 0·35	6·20 ± 0·30	4·61 ± 0·26	3·89 ± 0·28
Myofibril fragmentation index[a]	28·2 ± 0·9	38·3 ± 3·6	49·6 ± 3·4	55·4 ± 3·0	71·2 ± 2·6	72·4 ± 4·4
CDP-I[b]	64·1 ± 6·5	—	29·9 ± 2·7	—	28·7 ± 1·4	21·5 ± 3·2
CDP-II[c]	86·6 ± 2·0	—	86·6 ± 2·5	—	84·9 ± 4·5	76·7 ± 6·7
Inhibitor	−123·4 ± 5·5	—	−25·1 ± 1·2	—	−13·8 ± 2·9	−14·0 ± 2·2
Collagen						
Amount (mg/g)	—	—	3·67 ± 0·13	—	—	3·64 ± 0·12
Solubility (%)	—	—	11·48 ± 0·70	—	—	11·33 ± 0·65

[a] Absorbance at 540 nm × 200.
[b] Low Ca^{++}-requiring Ca^{++}-dependent protease A_{278}/200 g of muscle (caseinolytic activity).
[c] High Ca^{++}-requiring Ca^{++}-dependent protease A_{278}/200 g of muscle (caseinolytic activity).
* $n = 15$ for all measurements, except for CDP-I, -II and inhibitor at day 0, and day 14 ($n = 7$).

Inhibitor activity was then determined according to the following formula:

$$\text{Inhibitor activity} = (c - b) - (a) \times \text{dilution factor}$$

Myofibril fragmentation index (MFI)

MFI was determined at 0, 1/2, 1, 3, 6 and 14 days of post-mortem storage according to the procedure described by Olson *et al.* (1976).

Amount and solubility of collagen

Collagen content and solubility was determined after 1 and 14 days of post-mortem storage according to Bergman & Loxely (1963) and Hill (1966).

RESULTS

Effect of post-mortem storage on CDP-I, II and their inhibitor

The elution pattern of the DEAE-Sephacel column (Fig. 1) shows that this first step was adequate for separation of CDP-I (Peak II), CDP-II (Peak III) and their inhibitor (Peak I). The elution pattern is similar to those reported previously (Dayton *et al.*, 1981; Vidalenc *et al.*, 1983; Ducastaing *et al.*, 1985). Table 1 and Figs 2 and 3 indicate the effect of post-mortem storage on the parameters studied during these experiments. Post-mortem storage seems to

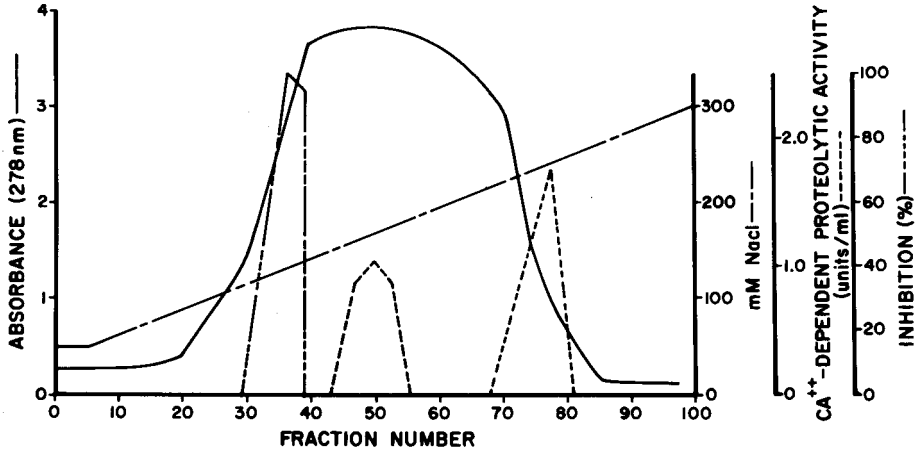

Fig. 1. Elution profile from DEAE-Sephacel of the 50 000 × g_{max} supernatant. The column was loaded with crude extract, washed with 5 volumes of elution buffer and the bound protein was eluted at 30 ml/h with a continuous gradient of NaCl from 50 to 500 mM in elution buffer. 8 ml fractions were collected.

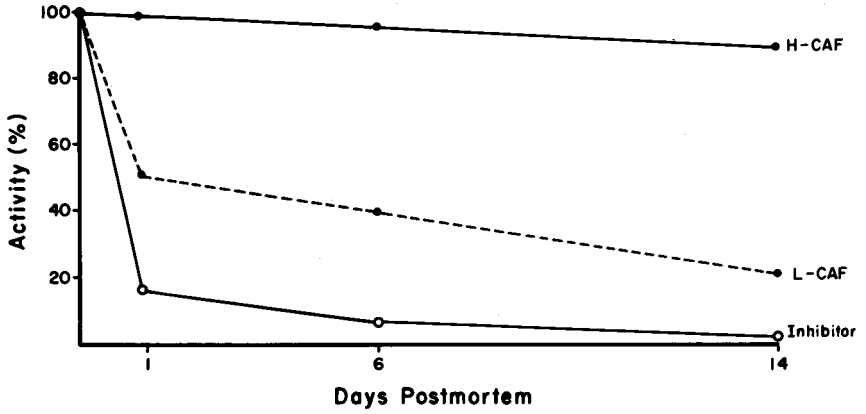

Fig. 2. Effect of post-mortem storage on the activities of two Ca^{++}-dependent proteases and their inhibitor. Beef carcasses were stored at 2°C for 14 days and experiments were conducted as described in the 'Materials and Methods' section. Calculations were made based on the assumption that maximum activity (100%) for proteases and inhibitor would be at 0 h.

have very little effect on the activity of CDP-II. Even after 14 days of post-mortem storage 80·2% of the original activity was present. Vidalenc *et al.* (1983) and Ducastaing *et al.* (1985) obtained the same results for rabbit and bovine skeletal muscle, respectively. However, CDP-I and inhibitor seem to lose their activity quite rapidly with post-mortem storage. According to our result, CDP's inhibitor appears to be the most susceptible to post-mortem storage, a conclusion which is in agreement with those reported by Vidalenc *et al.* (1983) for rabbit muscle. These results (i.e. susceptibility of inhibitor to post-mortem storage) do not agree with those reported by Ducastaing *et al.*

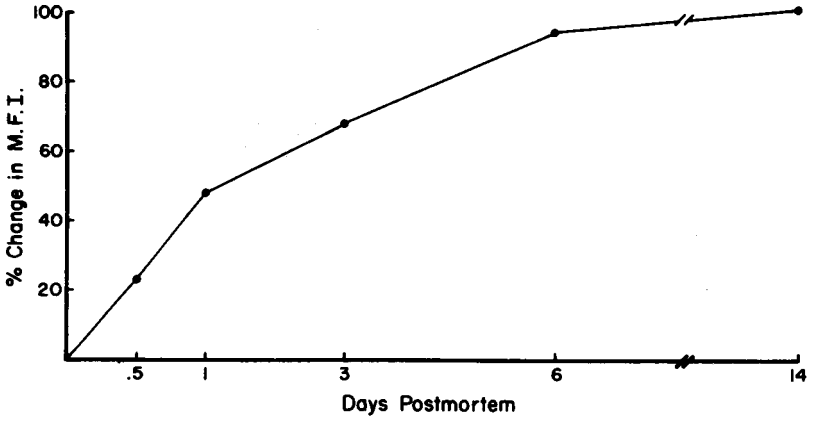

Fig. 3. Effect of post-mortem storage on the myofibrillar fragmentation index (MFI). Beef carcasses were stored at 2°C for 14 days and experiments were conducted as described in the 'Materials and Methods' section. Calculations were made based on the assumption that maximum MFI values (100%) would be observed on day 14.

(1985). Our results indicate that only 20·7% of the original inhibitor activity was present after 24 h and its activity was practically eliminated by 6 days of post-mortem storage. However, Ducastaing *et al.* (1985) reported that 90·0% of the inhibitor activity was present at 24 h and even after 72 h of post-mortem storage about 90% of its activity was preserved, a result which neither agrees with ours nor with Vidalenc *et al.*'s (1983) results. Because Ducastaing *et al.* (1985) reported only percentage of the activity and did not report the actual values (i.e. A_{278}/amount of muscle), it is impossible to explain this discrepancy. It is quite possible that Ducastaing *et al.* (1985) underestimated the activities at 0 h and consequently the activities during post-mortem storage is overestimated. Thus, we propose that in experiments of this kind the actual value (for comparative purposes) be reported rather than percentage alone.

Effect of post-mortem storage on the shear force, myofibril fragmentation and collagen

Results for shear force values are reported in Table 1. Because of the problems associated with cooking prerigor meat, shear force values were determined after 1, 3, 6 and 14 days of post-mortem storage. Results indicate that changes in shear values from day 1 to day 6 are the most dramatic, while those from day 6 to day 14 in shear values are reduced.

Myofibril fragmentation index (MFI) results are reported in Table 1 and Fig. 3 for actual values and also as a percentage of total improvement at any given day, respectively. These results indicate that almost 50% of the changes in the myofibrils have taken place by 24 h of post mortem, but it takes another 13 days for these changes to be completed. These results clearly demonstrate that post-mortem effects begin at the time of exsanguination. As such, we agree with Marsh *et al.* (1981) that quality enhancement is greatest and fastest during the very early post-slaughter period. The early post-slaughter period is also the period during which CDP-I is maximally active. Immediately after slaughter, the pH and temperature of muscle are 7·0 and 37°C, respectively (Fig. 4), and the ultimate pH and temperature are achieved gradually over a 24-h period. CDP-I is, therefore, expected to be more active during this period and capable of inducing maximum changes in the myofibrils as opposed to the period from 24 h to 14 days post mortem. It is quite interesting to note the remarkable resemblance between the activity profile of CDP-I and changes in the myofibril fragmentation throughout post-mortem storage. Results also indicated that post-mortem storage had no effect on the collagen in terms of amounts and its solubility. These results are in agreement with those reported previously (Pfieffer *et al.*, 1972; Pierson & Fox, 1976).

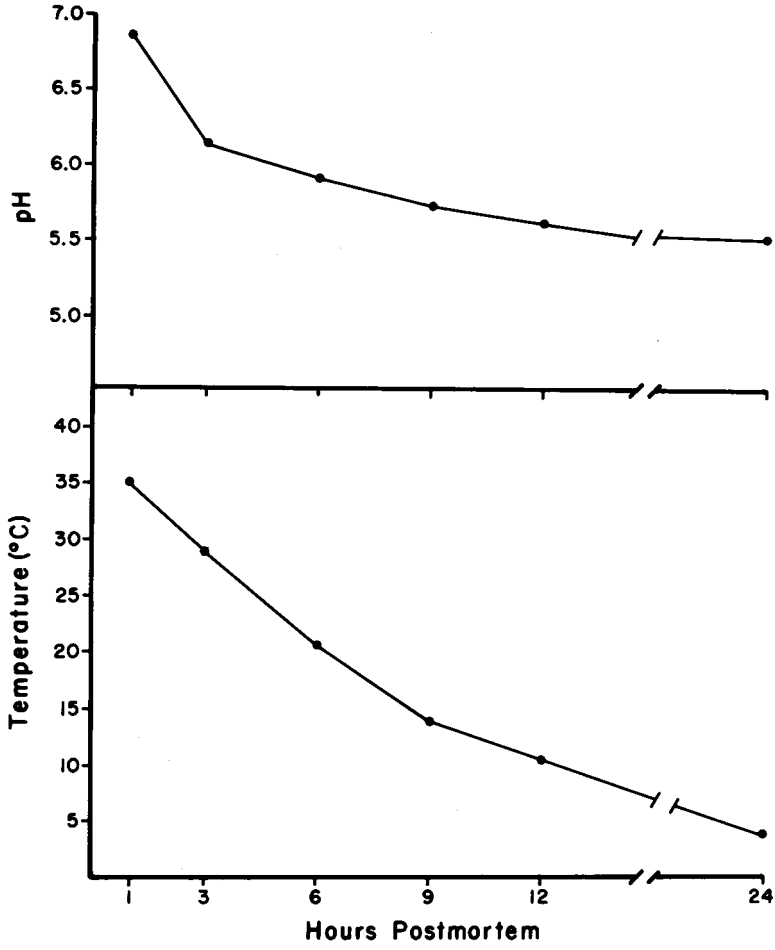

Fig. 4. Effect of post-mortem storage on the pH and temperature decline.

DISCUSSION

Clearly, the most dramatic changes in MFI are seen in the first 24 h with more gradual increases observed in MFI values from day 1 to day 6. From day 6 to day 14 little change in MFI values is seen. Paralleling the increases in MFI values, shear values from day 1 to day 6 are the most dramatic, and changes from day 6 to day 14 in shear values are, in turn, reduced.

Because of the demonstrated proteolytic specificity of both CDP's against myofibrillar proteins which ultimately creates myofibril fragmentation at the Z-disk (Koohmaraie *et al.*, 1984, 1986), it is reasonable to suggest a role for these proteases in the fragmentation process. Although the conditions of

pH and temperature (7·0 and 37°C) immediately post-slaughter would favor the activity of both proteases, the fact that only the activity of CDP-I changes after 24 h, the time interval that relates to the period of greatest myofibrillar fragmentation, suggests that only CDP-I may be involved in post-mortem tenderization. The rationale for this statement derives from the observation that both CDP's undergo autolysis in *in vitro* assays where sufficient Ca^{++} is available for CDP activation. Therefore, the rapid loss of CDP-I activity in 24 h may be a good indicator that CDP-I, unlike CDP-II, is activated under these post-mortem conditions and, once activated, is capable of hydrolyzing itself as well as myofibrillar proteins. Moreover, although CDP-II activity would also be favored under the early post-mortem conditions of pH and temperature, it is likely that free Ca^{++} levels ($\sim 10\ \mu M$) are not sufficient to activate CDP-II. This may also be the reason that CDP-II activity does not change with storage since autolysis of CDP-II would almost certainly require substantial amounts of Ca^{++}.

Reasons for the rapid loss of inhibitor activity are not immediately forthcoming. Studies investigating the interaction of a calcium-dependent protease inhibitor and CDP-II in bovine cardiac tissue have documented the fact that the inhibitor is highly susceptible to hydrolysis by CDP-II and that hydrolysis requires millimolar levels of calcium (Mellgren *et al.*, 1986). Whether CDP-I may be capable of hydrolyzing the inhibitor under the conditions existing immediately after exsanguination has not yet been determined. However, it is tempting to speculate that loss of inhibitor activity might be related to Ca^{++}-dependent proteolytic activity occurring at this time. Clearly, additional studies which would examine whether CDP-I, when activated in the post-mortem setting, is capable of hydrolyzing specific myofibrillar proteins, the endogenous inhibitor, and, perhaps, CDP-I itself, must be completed before the contribution of CDP-I in these circumstances can be understood.

CONCLUSIONS

The data presented here clearly demonstrate that post-mortem effects begin immediately after exsanguination, the period during which maximum myofibril fragmentation is observed. Because there were no detectable changes in the amount or solubility of collagen, it was concluded that the improvement in tenderness resulting from post-mortem storage at refrigerated temperatures must be derived from changes in the myofibrils. Since CDP-I activities parallel the myofibrillar changes, it is suggested that CDP-I may play a major role in the fragmentation of myofibrils and, consequently, in meat tenderization resulting from post-mortem storage.

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