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Calcium Chloride *in vitro* Effects on Isolated Myofibrillar Proteins

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ABSTRACT

The objective of this study was to determine the effect of 30 mM $CaCl_2$ on the solubilization of those structural proteins that contribute to myofibril stability. Ovine M. longissimus dorsi (longissimus) *samples were obtained immediately post-exsanguination, myofibrils were isolated, glycerated, and frozen until needed. Myofibrils were washed, diluted and incubated in 0·1 M KCl, 10 mM Tris, pH 7·0 buffer for 24, 48 and 72 h. Treatments consisted of: (1) control, (2) 1 mM E_{64}, (3) 30 mM $CaCl_2$, and (4) 1 mM E_{64} + 30 mM $CaCl_2$. Results (SDS-PAGE) indicated that myosin heavy chain (though not to a great extent), M-protein, C-protein, α-actinin, actin, troponin-T, tropomyosin isoforms, troponin-I and 72, 70, 62, 33, 32, 30, and 22 kDa unidentified bands were solubilized from myofibrils incubated in KCl buffer for 72 h. The addition of $CaCl_2$ hastened the appearance of some of the proteins in the supernatant fractions, but no differences were observed at 72 h among the treatments. The addition of E_{64} had no effect on which proteins were released. Thus, in the absence of proteolysis it appears that a general solubilization of thick-and-thin filament ancillary proteins occurs in the presence of 30 mM $CaCl_2$. However, the contribution to tenderness should be minimal, because solubilized proteins are not part of the cytoskeletal elements that are responsible for maintaining structural integrity of the tissue.*

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†Names are necessary in order to report factually on available data; however, the USDA neither guarantees nor warrants the standard of the product, and the use of the name by USDA implies no approval of the product to the exclusion of others that may also be suitable.

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INTRODUCTION

It has long been known that meat tenderness improves gradually as a result of post-mortem storage of carcasses at refrigerated temperatures (Lehmann, 1907). There is strong evidence that proteolysis of key structural myofibrillar proteins such as titin and desmin combined with weakening and/or degradation of Z-line by calpain is the major contributor to tenderization (Goll *et al.*, 1983; Koohmaraie 1988, 1992*a*,*b*). Calpains are proteinases having an absolute calcium requirement for proteolytic activity and are inhibited by zinc. Based on these facts, a process was developed to accelerate/enhance post-mortem tenderization by infusion of carcasses or injections of cuts of meat with a solution of $CaCl_2$ (for review, see Koohmaraie, 1988, 1992*a*,*b*). If $CaCl_2$ was added in the prerigor state, maximum tenderness and proteolysis of myofibrillar proteins were obtained 24 h post-mortem. The activation of calpains appears responsible for the observed effects because: (1) there was no μ-calpain activity remaining, and *m*-calpain and calpastatin activities were lowered in the carcasses infused with 0·3 M $CaCl_2$ (lower proteinase activity indicates activation of the proteinase and subsequent loss of activity through autolysis); (2) infusion of carcasses with NaCl at the same ionic strength as the $CaCl_2$ did not produce this tenderizing effect by 24 h; and finally, (3) infusion with $ZnCl_2$, a potent inhibitor of calpains, blocked the postmortem tenderization process (Koohmaraie, 1990). To further substantiate the argument that calpains are responsible for this tenderizing effect, in separate *in vitro* experiments, Koohmaraie *et al.* (1988*a*) demonstrated that incubation of muscle slices with 10 mM calcium chloride induces proteolysis of myofibrillar proteins and fragmentation of myofibrils. However, incubation of muscle slices with calcium chelators (EDTA or EGTA) prevents both degradation of myofibrillar proteins and myofibril fragmentation.

In spite of the overwhelming evidence in support of proteolysis as the mechanism of calcium chloride tenderization, it has been argued that proteolysis has no role in this process and that the changes are induced non-enzymatically by Ca^{2+} (Takahashi *et al.*, 1987). Taylor and Etherington (1991) suggested that the final concentration of Ca^{2+} obtained in the infused tissue (approximately 30 mM) was so high that the tenderization was due to two processes: proteolysis of myofibrillar proteins in or near the Z-line, and a calcium-dependent solubilization of other key myofibrillar proteins. Therefore, the objective of this study was to determine the effect of 30 mM $CaCl_2$ on the solubilization of those structural proteins that contribute to myofibril stability.

MATERIALS AND METHODS

Myofibrils

Ovine *M. longissimus dorsi* (*longissimus*) muscle samples were obtained immediately after exsanguination. Myofibrils were isolated according to the procedures of Goll *et al.* (1974*b*). Myofibrils were stored in 50% glycerol, 100 mM NaCl, and 1 mM sodium azide at –20°C. Myofibrils were thawed and centrifuged at 2000 x g_{max} for 15 min, followed by two 40-ml washes with 100 mM NaCl and a final 40-ml wash using the incubation buffer. Protein concentrations then were determined using the biuret procedure (Gornall *et al.*, 1949) using Bovine Serum Albumin (BSA)·as standard.

Incubations

Adequate volumes of myofibril suspensions were placed in 1·5-ml Eppendorf centrifuge tubes to give a final incubation protein concentration of 10 mg/ml. Suspensions were centrifuged at 6000 x g_{max} for 2 min at 4°C. Pellets were resuspended with 1 ml of cold 0·1 M KCl, 10 mM Tris/maleate, pH 7·0. Treatments included: (1) buffer control, (2) buffer + 1 mM *N*-(M-[L-3-trans-carboxyoxiran-2-carbonyl]-L-leucyl)-agmatine (E_{64}, an irreversible inhibitor of cysteine proteinases), (3) buffer + 30 mM $CaCl_2$ and (4) buffer + 1 mM E_{64} + 30 mM $CaCl_2$. Prior to the addition of $CaCl_2$, treatments were subjected to continuous mixing at 4°C for 0, 24, and 48 h. Once $CaCl_2$ was added, the treated myofibrils as well as controls were mixed continuously for an additional 24 h. Total incubation times were 24, 48, and 72 h. After incubation, myofibrils were centrifuged at 6000 x g_{max} for 1 min at room temperature and the myofibrillar pellets were prepared for SDS-PAGE. Supernatant fractions were recentrifuged at 10 000 x g_{max} for 10 min at room temperature. Proteins in aliquots of the second supernatant fractions were precipitated with an equal volume of 100% ethanol (–20°C), centrifuged at 10 000 x g_{max} for 5 min, and precipitates were prepared for SDS-PAGE.

SDS-PAGE

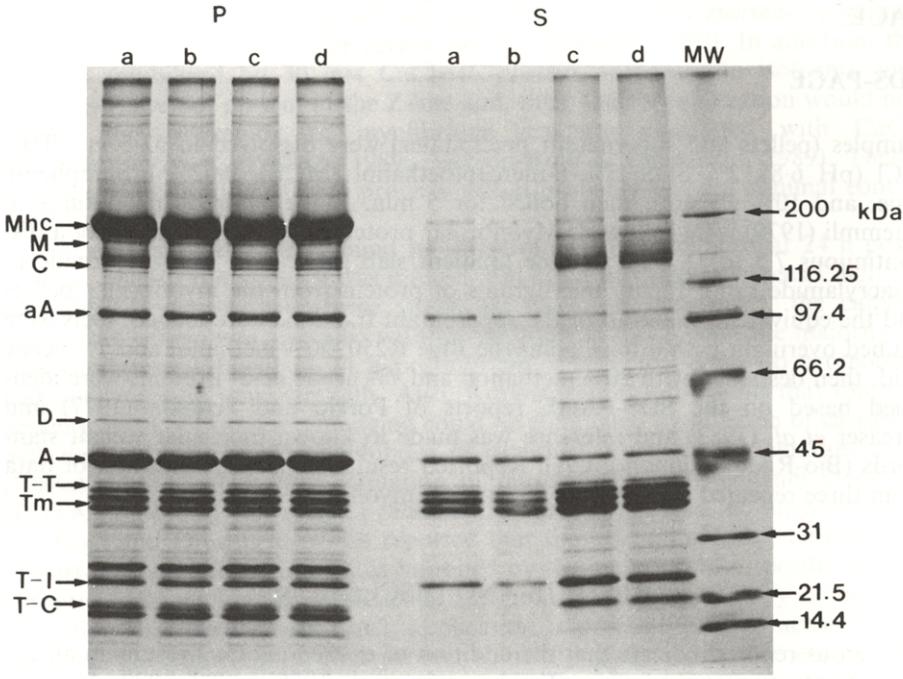
Samples (pellets and supernatant precipitates) were dissolved in 62·5 mM Tris-HCl (pH 6·8), 2% SDS, 5% β-mercaptoethanol (MCE), 0·02% bromophenol blue, and 10% glycerol, then boiled for 5 min. Electrophoretic procedures of Laemmli (1970) were followed. Myofibrillar proteins were separated using a discontinuous 7·5 to 15% acrylamide gradient slab gel with a 75:1 acrylamide to bisacrylamide ratio. Eighty micrograms of protein from the myofibrillar pellets and the equivalent of 100 μl of the supernatant fractions were loaded. Gels were stained overnight with 0·1% Coomassie Blue R250, 50% methanol and 7% acetic acid, then destained with 20% methanol, and 7% acetic acid. Proteins were identified based on the SDS-PAGE reports of Porzio and Pearson (1977) and Greaser *et al.* (1983) and reference was made to known molecular weight standards (Bio-Rad, Richmond, CA). Reported results are a representation of data from three repeated experiments of separate myofibril extractions.

RESULTS AND DISCUSSION

Numerous reports indicate that the addition of exogenous Ca^{2+} results in an enhancement of meat tenderness (Koohmaraie *et al.*, 1988b, 1989, 1990; Alarcon-Rojo & Dransfield, 1989; Koohmaraie & Shackelford, 1991; Morgan *et al.*, 1991; Wheeler *et al.*, 1991; Whipple & Koohmaraie, 1993). This tenderness improvement seems to be through the activation of the calpain proteinases, because they degrade those proteins (titin, desmin and troponin-T) that are hydrolyzed in fully-conditioned meat (Goll *et al.*, 1983; Koohmaraie, 1988; Whipple and Koohmaraie, 1991; Kendall *et al.*, 1993). Also, disassembly of the Z-line is associated with tender meat (Goll *et al.*, 1974a), and reports implicate calpain involvement in this process (Koohmaraie *et al.*, 1986; Slinde & Kryvi,

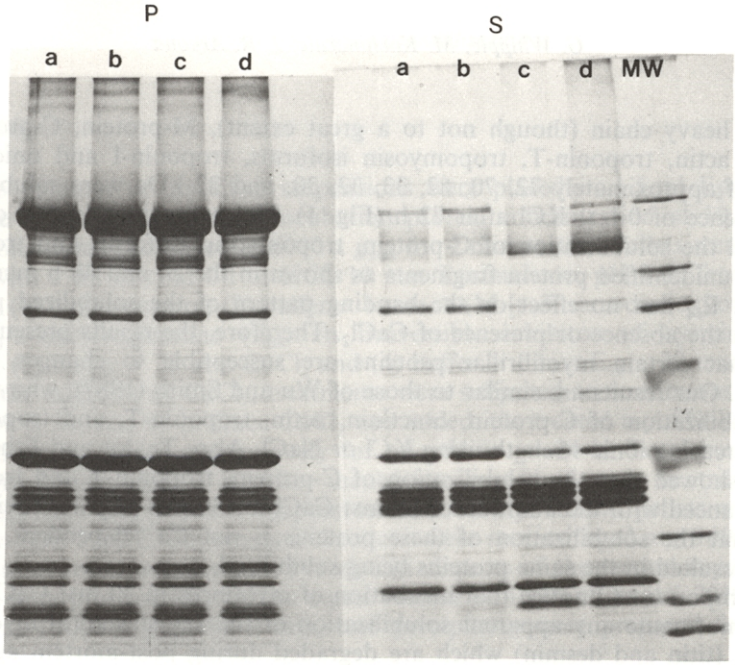
1986). However, it remains controversial as to the extent of protein solubility and its contribution to the tenderness improvement with $CaCl_2$ application. Therefore, in this study we employed a 30 mM $CaCl_2$ concentration [the concentration found in the $CaCl_2$ infused or injected tissue (Koohmaraie *et al.*, 1988*a*, 1989, 1990; Koohmaraie and Shackelford, 1991)] with the objective of determining if those proteins solubilized by 30 mM $CaCl_2$ in the absence of proteolysis are those which are commonly associated with improvement in tenderness. To avoid any contamination from residual calpain, myofibrils were extracted with Triton X-100 (Goll *et al.*, 1974*b*) to facilitate calpain removal (Mellgren *et al.*, 1982). Precautions were also taken to suppress any calpain activity by conducting incubations at 4°C in the absence of the reducing agent, MCE (Jiang *et al.*, 1991). In addition, the cysteine proteinase inhibitor, E_{64}, which irreversibly inhibits calpain activity in the presence of Ca^{2+}, was added to the incubation mixture.

Results from SDS-PAGE of the myofibrillar pellets indicated no treatment differences by 72 h (Fig. 1). Evaluation of the supernates indicated that 30 mM $CaCl_2$ had no additional effect as to which myofibrillar proteins were solubilized when compared to 100 mM KCl buffer incubation for the 72 h duration (Fig. 1).

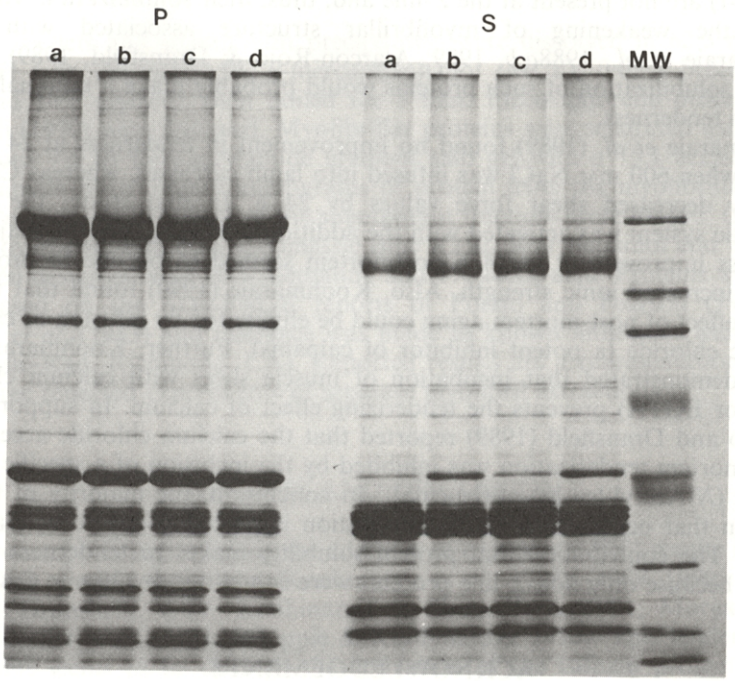


(a)

Fig. 1. SDS-PAGE of myofibril pellets (P) and supernatant fractions (S) for (a) 24 h incubations, (b) 48 h incubations, and (c) 72 h incubations. Lane identification: a, buffered control; b, buffer + E_{64}; c, buffer + $CaCl_2$; d, buffer + E_{64} + $CaCl_2$; MW, molecular weight standard given in kDa. Myofibrillar proteins abbreviated Mhc (myosin heavy chain), M (M-protein), C (C-protein), aA (α-actinin), D (desmin), A (actin), T-T (troponin-T), T-I (troponin-I) and T-C (troponin-C).



(b)



(c)

Fig.1.—*contd.*

Myosin heavy chain (though not to a great extent), M-protein, C-protein, α-actinin, actin, troponin-T, tropomyosin isoforms, troponin-I and unidentified bands of approximately 72, 70, 62, 33, 32, 30, and 22 kDa were solubilized in the presence of 0·1 M KCl after 72 h (Fig. 1). However, the addition of $CaCl_2$ hastened the solubilization of C-protein, tropomyosin isoforms and troponin-I and the unidentified protein fragments as shown in the 24 and 48 h incubations (Fig. 1). E_{64} had no effect on the banding pattern of the solubilized proteins, either in the absence or presence of $CaCl_2$. Therefore, the results presented here show that certain myofibrillar proteins are susceptible to changes in ionic strength. Our results are similar to those of Wu and Smith (1987), who reported the solubilization of C-protein, α-actinin, actin, troponin-T, and tropomyosin with increasing ionic strength using KCl or NaCl. Also, Taylor and Etherington (1991), showed that the solubilization of C-protein, troponin-T and troponin-I was enhanced with the addition of 30 mM $CaCl_2$ or 30 mM $MgCl_2$. Their results show that the solubilization of these proteins is not Ca^{2+}-dependent, because $MgCl_2$ resulted in the same proteins being solubilized.

It is important to notice that incubation of myofibrils in 30 mM $CaCl_2$ at pH 7·0 did not cause any apparent solubilization of the key-structural myofibrillar proteins (titin and desmin) which are degraded during post-mortem aging and are hydrolyzed by calpain (for review, see Koohmaraie, 1992). In addition, the proteins solubilized by 30 mM $CaCl_2$ (C-protein, tropomyosin isoforms, and troponin-I) are not present at the Z-line and, thus, their solubilization would not explain the weakening of myofibrillar structure associated with $CaCl_2$ (Koohmaraie *et al.*, 1988*a*,*b*, 1989; Alarcon-Rojo & Dransfield, 1989). Therefore, the solubilization of such proteins would probably make a minimal contribution to tenderness.

Koohmaraie *et al.* (1989) found no improvement in tenderness at 24 h post-mortem when 600 mM NaCl was infused into lamb carcasses, whereas $CaCl_2$ remarkably decreased shear force values by 24 h. In that study, the calpain proteolytic system was activated with the addition of $CaCl_2$, thus the majority of tenderness improvement at 24 h postmortem seemed to be due to proteolysis and not increased ionic strength. Also, Koohmaraie (1990) found that the tenderizing effect of post-mortem aging could be eliminated by infusion of carcasses with zinc chloride (a potent inhibitor of calpains). Further, Koohmaraie *et al.* (1988*a*) demonstrated that incubation of muscle slices with calcium chelators (EDTA or EGTA) prevents the tenderizing effect of calcium. In support, Alarcon-Rojo and Dransfield (1989) reported that the calcium chloride acceleration of post-mortem tenderization was inhibited by the inclusion of a specific calpain inhibitor (*N*-Acetyl-leu-leu-norleucinal). In conclusion, the majority of the tenderization that occurs with $CaCl_2$ application is probably due to activation of calpains. The contribution that protein solubility makes to tenderness may be minimal because only non-Z-line proteins were solubilized by 30 mM $CaCl_2$.

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