THE INFLUENCE OF pH, Ca^{2+} CONCENTRATION AND TEMPERATURE ON THE PROTEOLYTIC ACTIVITY ON MYOFIBRILS OF CANP (Ca^{2+}-ACTIVATED NEUTRAL PROTEINASE) ISOLATED FROM LAMB SKELETAL MUSCLE.

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Summary.- The two calcium-activated neutral proteinases (µCANP and mCANP) and their specific inhibitor were isolated by ion exchange chromatography in DEAE-Sephacel from lamb skeletal muscle (*Longissimus dorsi*). Their proteolytic activities were then determined using myofibrils as substrate. Ca^{2+}-activated neutral protease showed a relevant activity in the range 5.5-6.5 (≥40% of maximum activity at pH 7.5). Ca^{2+}-requirements were different for each form of the enzyme, µCANP needed 50 µM Ca^{2+} for half-maximal activity, while the other isoenzyme, mCANP, needed 1,000 µM Ca^{2+} for reaching 50% of its maximum activity. With regard to the effect of temperature, both isoenzymes retained ≥25% of their activity at 25°C with a temperature reduction down to 4°C. In conclusion, µCANP is an active protease in conditions similar to that prevalent in meat during postmortem storage.

INTRODUCTION.

Post-mortem storage of carcasses at refrigerated temperature has been known to improve meat tenderness for many years and still remains an important procedure for producing tender meat. There is general agreement that proteolysis of myofibrillar proteins is the major contributor to meat tenderization during postmortem storage (Goll et al 1983a).

Since Busch et al. (1972) reported the evidence that Ca^{2+}-activated neutral protease (CANP) had the ability to degrade z-disks, many investigators supported its likely role in the postmortem tenderization process of meat (Dayton et al, 1976; Elgasim et al, 1985; Koohmaraie et al 1987). This protease received many different names; being most relevant: calcium-activated sarcoplasmic factor (CASF), calcium activated factor (CAF), calcium-activated neutral protease (CANP, which included a low-calcium-requiring form, µCANP, and a high-calcium-requiring form, mCANP), and calcium-dependent proteinase (CDP; including a low-calcium requiring form, CDP I, and a high-calcium requiring form, CDP II). Both proteases are also called calpains, while the specific inhibitor is called calpastatin.

Until 1980 one of the major obstacles to understanding the physiological role of the calcium-activated protease has been the fact that the purified enzyme required 1-2 mM calcium in order to express maximum activity. Mellgren, in 1980, showed that canine skeletal muscle contained; besides the high-calcium requiring

(mCANP) form, a low-calcium-requiring form (µCANP); the low-calcium requiring protease had detectable activity at calcium concentrations as low as 5 µM, while the high calcium-requiring protease did not have detectable activity until calcium concentration exceeded 0.1 mM. Both proteases required for maximum activity a neutral pH (Dayton et al, 1976) and had little activity at pH 5.5-5.8. On the basis of these arguments, it seems unlikely that mCANP could be responsible for postmortem changes. If CANP is to be involved, it is the low-Ca^{2+}-requiring form of CANP, due to its low calcium requirements, which ought to be responsible.

Additionally, a specific inhibitor of the calcium-activated neutral proteinase was found in various tissues (brain, lung, heart, liver; Murachi et al, 1981). The simultaneous presence of CANP in these tissues indicates that the physiological role of this enzyme seems to be closely controlled.

We report here the isolation from lamb skeletal muscle of both the low-calcium-requiring and high-calcium-requiring proteases and their specific inhibitor, as well as the study of the influence of conditions prevalent during postmortem aging of meat (ie. pH ≥5.5; 4°C; ≈50 µM Ca^{2+}) on µCANP and mCANP activity. We have used myofibrils throughout as substrate since CANP activity on casein has been extensively studied by other authors, while myofibrillar proteins have a higher interest as they are directly implicated in the postmortem tenderization process.

MATERIALS AND METHODS.

Preparation of calcium-dependent proteases and their inhibitor. Low-calcium-requiring and high-calcium-requiring forms and their inhibitor were isolated from 400 g lamb skeletal muscle (*Longissimus dorsi*) according to the procedure described by Koohmaraie et al (1988). Briefly, chilled muscle was ground, suspended in 2.5 volumes of 10 mM tris-HCl, pH 7.5, containing 4 mM EDTA, 50 mM NaCl and 2 mM 2-mercaptoethanol (MCE), homogenized in an Ultraturrax and centrifuged a 15,000xg for 40 min. The resulting supernatant was filtered through two layers of cheese cloth and its pH was adjusted to 7.5 followed by centrifugation at 30,000xg for 50 min. The supernatant was filtered through glass wool and applied to a DEAE-Sephacel Column that had been equilibrated with 5 mM tris-HCl, pH 7.5, containing 50 mM NaCl, 0.1 mM EDTA, 2 mM MCE. Columns were then washed with the same buffer to remove unbound protein, until absorbance of the outflow at 278 nm was between 0.1 to 0.4. The bound proteins were then eluted with a continous gradient of 50-500 mM NaCl in 5 mM tris-HCl, pH 7.5, containing 0.1 mM EDTA and 2 mM MCE.

Protein concentration. Protein concentrations were determined according to Lowry et al (1951).

Myofibril isolation. Myofibrils were prepared immediately after slaughter from *Longissimus dorsi* muscle following the procedure described by Olson et al (1976), by using 100 mM KCl, 20 mM PO_4H_2K, 0.1 mM EDTA, 1 mM Cl_2Mg, 1 mM NaN_3, as the isolating medium. Protein concentration was determined by the Nessler method (Johnson, 1941). Freezing was carried out in liquid nitrogen.

Assay for CANP activity. Calcium-dependent proteolytic activity was assayed, according to the procedure described by Koohmaraie et al (1986), using either casein (Hammerstein) or purified myofibrils as substrate at 25°C (or stated temperature) in 10 mM KCl, 50 mM tris-acetate, pH 7.5 (or stated pH), 10 mM MCE, 2.5 mM Ca^{2+} (or stated Ca^{2+}) and 5 mg/ml casein or 10 mg/ml myofibril protein. Total reaction volume was 2 ml. Control for enzyme as substrate accompanied each assay. The reaction was initiated by addition of CANP and stopped by addition of 2 ml of 5% trichloroacetic acid (TCA) when casein was used or 0.22 ml of 100 mM EDTA when myofibrils were used.

The assay tubes were then centrifuged at 1,000xg for 15 min (when myofibrils were used centrifugation was 9,500xg for 30 min) and the absorbance of the supernatant was measured at 278 nm.

To assess the effects of calcium concentration on the CANP activity, either low-calcium-activated or high-calcium-activated proteases were extensively dialyzed against the same buffer employed to assay their activity.

Assay for inhibitor activity. The activity of the inhibitor was determined by pre-incubating appropriate amounts of inhibitor and enzyme at 25°C for 60 min in 1.5 ml reaction mixture (Koohmaraie et al, 1986). For each fraction the following measurements were made:

1. Enzyme alone in calcium-containing reaction mixture (to determine calcium-dependent caseinolytic activity).
2. Fraction being assayed alone in EDTA-containing reaction mixture (to determine calcium-independent activity).
3. Enzyme + inhibitor in calcium-containing reaction mixture.

Inhibitor activity was then determined according to the following formula: (3-2) - (1)x dilution factor.

Inhibitor activity is reported as total absorbance units at 278 nm/400 g muscle in the caseinolytic assay.

RESULTS AND DISCUSSION.

Isolation of calcium-activated neutral protease. As shown in figure 1, two peaks of calcium-activated proteolytic activity (peaks II and III) and a peak of inhibitory proteolytic activity (peak I), were eluted from the 3.5x60 cm DEAE-Sephacel column to which the crude calcium-activated protease had been bound. Protease activity was obtained using casein as substrate and inhibitory activity was calculated by adding a fixed amount of inhibitor from each eluted fraction to a given enzyme activity.

Peak III (mCANP) calcium-activated proteolytic activity appeared to be very similar to the calcium-activated proteolytic activity isolated from skeletal muscle tissue by Busch et al (1972), and peak II (μCANP) activity was very similar too to the isolated from porcine skeletal muscle by Dayton et al (1976, 1981). The elution pattern showed almost no differences with those reported previously for other animal species (Dayton et al, 1981; Vidalenc et al, 1983 and Koohmaraie et al, 1987).

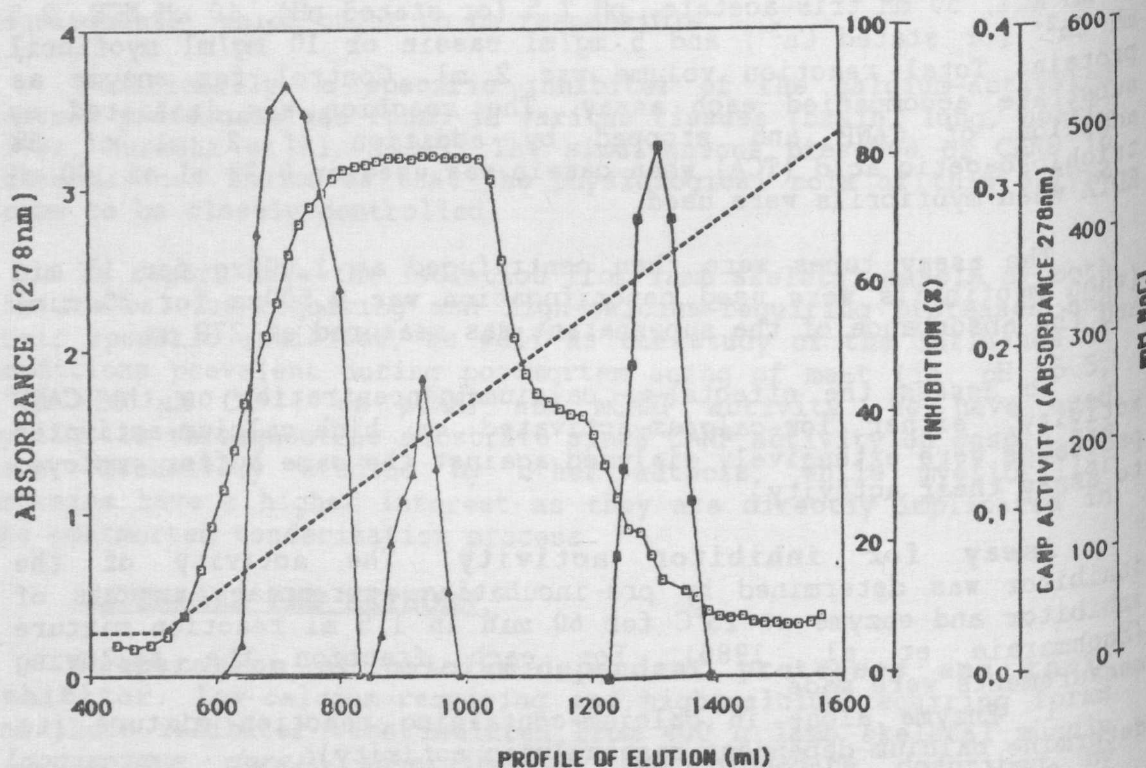

Figure 1.–Elution profile from DEAE-Sephacel of the 30,000xg supernatant. The column was loaded with 13,077 mg of crude extract, washed with 2 volumes of elution buffer and the bound protein was eluted at 60 ml/h with a 2,000 ml continous gradient of NaCl from 50 to 500 mM in elution buffer; 9 ml fractions were collected. □ absorbance at 280 nm; △ inhibitor (peak I); ▲ μCANP (peak II); ■ mCANP (peak III).

Calcium requirements of isolated μCANP and mCANP. The concentration of calcium required to activate μCANP and mCANP proteases was examined using myiofibrils as substrate. Although both μCANP and mCANP are activated by calcium, we found (Figure 2) μCANP to possess activity at substantially lower levels of calcium than does mCANP protease. With our assay conditions, isolated μCANP showed half-maximal activity at a calcium concentration as low as 50 μM and possessed detectable activity even at 10 μM calcium. In contrast, mCANP protease showed half-maximal activity at 1,000 μM calcium and no activity could be detected in the presence of a calcium concentration lower than 500 μM.

These results are in agreement with those of other investigators (Dayton et al, 1981; Pontremoli et al, 1984; Koohmaraie et el, 1986), who reported for other animal species that low-calcium-requiring form (µCANP) was activated by micromolar Ca^{2+}: 1-50 µM, while the high-calcium-requiring form (mCANP) needed 1-5 mM Ca^{2+} for activation.

The existence of a form of calcium activated protease that is active at calcium concentrations approaching those known to be present in the sarcoplasm of skeletal muscle cell is extremely important, since it is this form of the protease which can be active in vivo or in conditions prevalent in postmortem storage of muscle.

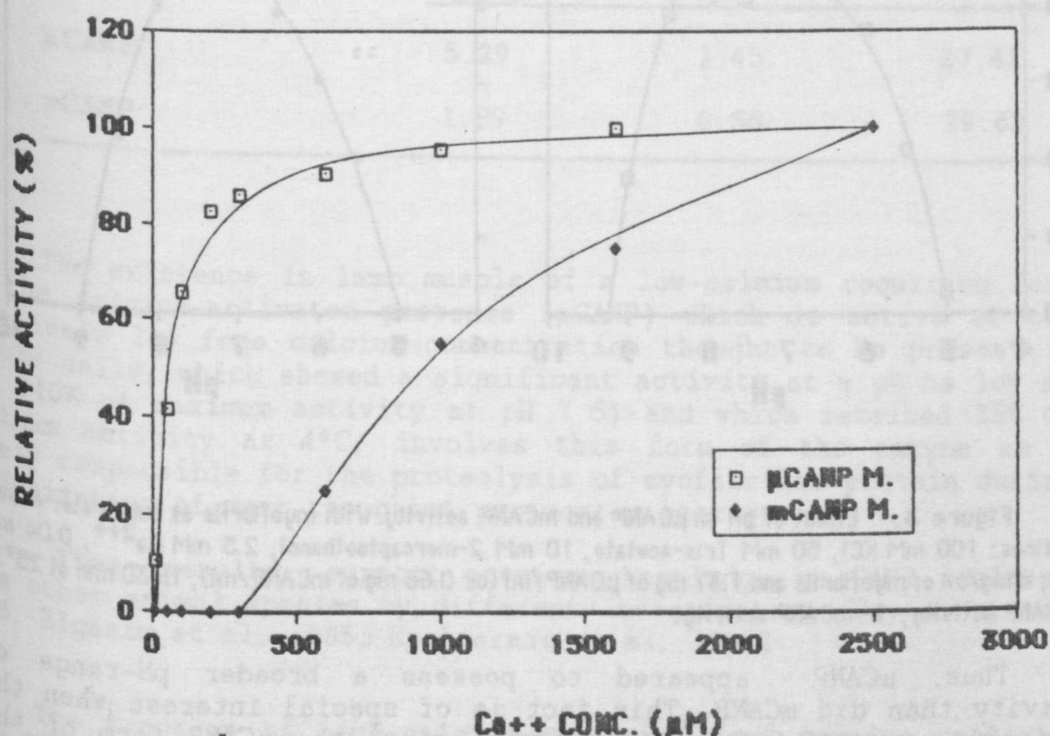

Figure 2.- Effect of Ca^{2+} concentration on µCANP and mCANP activity with myofibrils as substrate. Assay conditions: 100 mM KCl, 50 mM Tris-acetate, 10 mM 2-mercaptoethanol, 0.04 mM EDTA, 8 mg/ml of myofibrils and 0.112 mg of µCANP (or 0.05 mg of mCANP/ml) at 25°C, 1h 30 min.

pH dependency of µCANP and mCANP proteolytic activity. As shown in figure 3, the pH dependency of isolated µCANP and mCANP was found to be similar to that of the calcium-activated protease previously purified by Dayton et al (1981). Maximum proteolytic activity against a myofibrillar substrate was observed between pH 6.5 and 7.5, with reduced activity below pH 6.0 or above pH 8.5. Nevertheless, both µCANP and mCANP retained significant activity at a pH as low as 5.5 (40% of maximum activity at pH 7.5) and µCANP retained activity at a pH as high as 8.5 (75% of maximum activity),

while mCANP retained only 45% of its maximum activity at this same pH. Furthermore μCANP showed 5% of its maximum activity at pH 5 while mCANP retained no activity at this low pH.

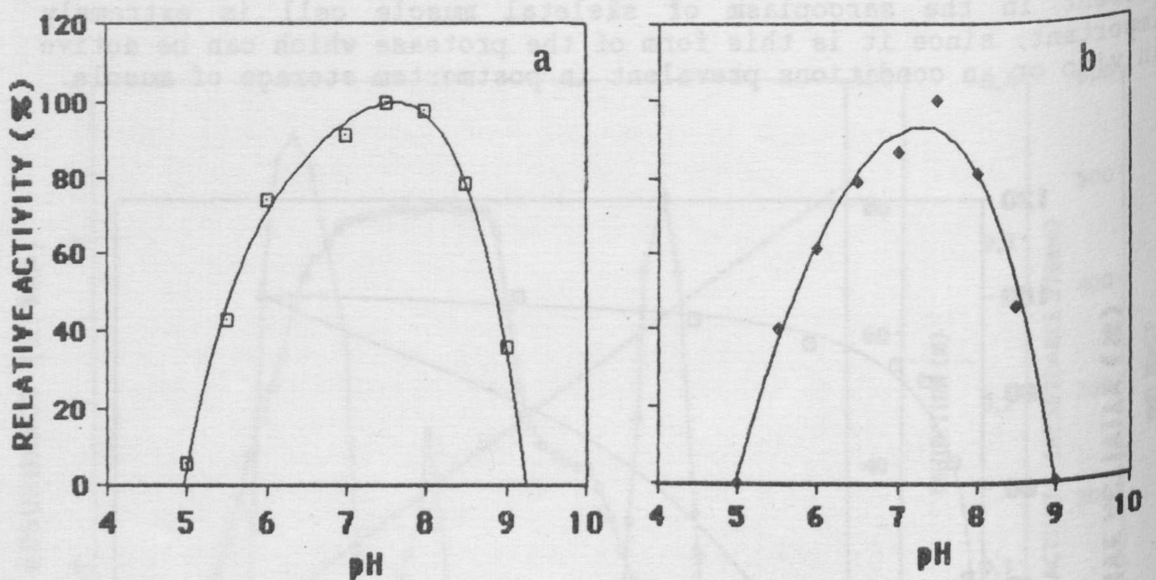

Figure 3.– Effect of pH on μCANP and mCANP activity with myofibrils as substrate. Assay conditions: 100 mM KCl, 50 mM Tris-acetate, 10 mM 2-mercaptoethanol, 2.5 mM Ca^{2++}, 0.04 mM EDTA, 8 mg/ml of myofibrils and 1.37 mg of μCANP/ml (or 0.66 mg of mCANP/ml), 1h 30 min at 25° C. **a**: μCANP activity; **b**: mCANP activity.

Thus, μCANP appeared to possess a broader pH-range of activity than did mCANP. This fact is of special interest when the pH in the range 5.5-6 is considered, since the μCANP form of the enzyme has shown to maintain enough activity for degrading myofibrils under conditions which prevale in muscle after slaughter. This agrees with all previous studies on CANP sensibility to pH, in which results similar to ours have been reported (Busch et al, 1972; Dayton et al, 1976 and Koomaraie et al, 1986).

Temperature dependency of μCANP and mCANP proteolytic activity. As is evident the low-calcium requiring and the high-calcium-requiring form of the protease are more active at 25°C than at 4°C, but they retained even 25% of maximun activity at 4°C (Table 1), which agrees with results obtained by Koohmaraie et al (1986). Thus, the enzymes retained a significant activity even at the low temperatures usually required for conditioning and aging of meat.

Table 1.- Effect of temperature, (4°C and 25°C) on µCANP and mCANP activity with myofibrils as substrate. Assay conditions: 100 mM KCl, 50 mM Tris-acetate, 10 mM 2-mercaptoethanol, 0.04 mM EDTA, 8 mg/ml of myofibrils and 0.055 mg of µCANP/ml (or mCANP), 1h 30 min. incubation.

	Enzyme activity		
	Absorbance/mg protein		%
	25ºC	4ºC	
µCANP	5.29	1.45	27.41
mCANP	1.89	0.56	29.62

The existence in lamb muscle of a low-calcium requiring form of the calcium-activated protease (µCANP) which is active at the relatively low free calcium-concentration thought to be present in muscle cells, which showed a significant activity at a pH as low as 5.5 (40% of maximum activity at pH 7.5) and which retained 25% of maximum activity at 4ºC, involves this form of the enzyme as a likely responsible for the proteolysis of myofibrilar protein during tenderization of meat thoughout postmortem storage.

These results support previous knowledge on CANP isolated from other animal species by different investigators (Dayton et al, 1976; Elgasim et al, 1985; Koohmaraie et al, 1987).

The function of high-calcium-requiring form remains unclear. This form of the protease can be converted by limited autolysis to a low calcium-requiring form (Dayton et al, 1981). If one takes into account this hypothesis the high-calcium-requiring form could be a reserve of the calcium-activated protease in muscle, essentialy in those tissues where there is not a low-calcium requiring form; also during postmortem storage when the high-calcium-requiring form keeps a high activity (99%) during more than forteen days, while the low-calcium-requiring form loses 50% of maximum activity at 24h postmortem (Koohmaraie et al 1987).

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