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Muscle Ultrastructural Changes by Lysosomal Enzymes

1. Transmission Electron Microscopic Studies

by

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Lysosomal Enzyme에 依한 筋肉組織의 變化

第1報 Transmission Electron Microscopy에 의한 考察

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Abstract

Ultrastructural changes in Z-line, M-line and myofilaments of bovine *psoas* muscle produced by leukocyte lysosomal enzymes *in vitro* at different pH values (pH 7.0 and 4.0), temperatures (37 and 4°C) and time intervals (12, 24 hours at 37°C and 36, 168 hours at 4°C) were studied by transmission electron microscope. Muscle incubated with leukocyte lysosomal enzymes at pH 7.0 produced distinguishable degradation of Z-line, M-line and H-zone at both temperatures but at acidic pH (pH 4.0), Z-line were very stable and myofilaments were severely disintegrated.

Introduction

Textural changes associated with tenderization of meat during postmortem aging of beef are influenced by many factors (Macfarlane *et al.*, 1974). The tenderizing effect is mainly due to partial degradation of the myofibrillar components of muscle as a result of weakening at the junction of the actin filaments with the Z-lines (Davey and Dickson, 1970; Bouton and Harris, 1972).

Connective tissue strength is affected negligibly while the fiber strength is reduced considerably. Lysosomal hydrolases are strongly implicated in the tenderization of beef during aging, since the conditions prevailing in the meat during the aging process is favorable for hydrolase activities. The exact role and extent of involvement, and the major source of these lysosomal hydrolases are not fully established. Earlier reports from our laboratory suggest that these hydrolases could come from the leukocytes of the residual blood in the meat and that these proteolytic enzymes degrade purified myofibrillar protein (Venugopal, 1970; Kim, 1974).

Despite good bleeding, about 50% of total blood remains in the carcass as residual blood. Unbled muscles under sterile conditions undergo tenderizing changes to a greater extend than those which are bled. To investigate the action of purified porcine leukocyte lysosomal hydrases on thin strips of bovine *psoas* muscle and on isometric tension by physiograph, the ultrastructural changes were observed by transmssion electron microscope (TEM).

Materials and Methods

Enzyme Preparation

Leukocyte lysosomal enzyme mixture was prepared from porcine leukocytes by the procedure of Venugopal (1970). About one liter of porcine blood was collected asceptically in 15ml of 5% EDTA in 1% sodium chloride in a 5 : 1 ratio and allowed to settle. The leukocytes were spun down at 250xg for 10 minutes from the leukocyte rich plasma layer at 4°C. Residual erythrocytes were lysed by washing the leukocyte pellet with 0.25% sodium chloride. The leukocytes were finally washed once with cold 0.25M sucrose which contained 0.01M EDTA adjusted to pH 7.2. The leukocytes, suspended in sucrose, were sonicated in ice cold water bath. The sonicated leukocytes were then subjected to 3 or 4 strokes in Elvejhem homogenizer and centrifuged at 500xg for 20 minutes to remove cellular debris and any intact cells. The supernatent was centrifuged at 10,000x*g* for 30 minutes to sediment the lysosomes. The lysosomal pellet was frozen in dry ice and ground with chilled 4% aqueous 1-butanol. The aqueous layer was separated, using a separatory funnel. The butanol extract was dialyzed againt distilled water for 24 hours at 4°C. The dialyzate was lyophilized and used as enzyme source.

Muscle Preparation

Fresh bovine *psoas* muscle was obtained immediately after slaughter from the University of Missouri Abattoir. The muscle was brought immediately to the Meat Laboratory where they were freed of excess fat and connective tissue using sterile equipment. Sterile procedures were used to cut 2×2×2mm block of tissue from the internal area of the muscle. These sections were treated immediately to determine the influence of enzyme treatment on the microstructure of the muscle.

Treatment of Muscle Strips with Enzymes

The effect of this lysosomal enzyme preparation on the ultrastructure of the muscle strips was studied at pH 4.0 and 7.0, at 4 and 37°C and four time intervals(12, 24 hours at 37°C and 36, 168 hours at 4°C). Fifteen pieces of muscle strips (2×2×2mm) were mixed in a 10ml specimen bottle with 1ml of enzyme solution (mg enzyme protein/ml of deionized water) and 1 ml of buffer solution (0.1M acetate, pH 4.0 and 0.05M phosphate, pH 7.0) containing 2mM NaN_3. Control samples contained 1ml of deionized water instead of enzyme. Incubation at 37±0.5°C was done an incubator for the specified time period and incubation at 4±1°C was done in the refrigerator.

Electron Microscopy

Following incubation, the muscle pieces were washed with 1.25% glutaraldehyde buffered with 0.07M NaH_2PO_4 and 0.041M Na_2HPO_4 (pH 7.3) and 0.043M NaCl(Sjöstrand, 1967) and then fixed in a fresh solution of glutaraldehyde for 2 hours at 4°C. After washing overnight with the phosphate buffer (pH 7.3), the samples were post-fixed in 1% osmium tetraoxide for 2 hours at 4°C. The samples were dehydrated in acetone (20, 40, 60, 80 and 100%×3) and then infiltrated in spurr (acetone: spurr=3 : 1, 1 : 1, 1 : 3 and 100% spurr). Infiltrated samples were embedded in spurr and polymerized for 48 hours at 65°C and sectioned on a Richert Ultramicrotome. Silver sections (approximately 60-80nm) were deposited on uncoated cupper grid (300 mesh), stained with uranyl acetate (Sjöstrand, 1967) and lead citrate (Reynolds, 1963) and examined under RCA 3Gtransmission electron microscope at 100KV as accelerating voltage.

Isometric Tension Measurement

Isometric tension was measured by the procedure of Busch *et al.*(1972). Fresh bovine muscle obtained immediately after slaughter were cut into thin strips (0.5×0.5×7cm) paralleled to the longitudinal axis of the muscle fibers. One end of the strip was attached to an isometric myograph transducer (E & M Instrumental Co., Houston, Texas) and the other end was tied to a glass rod at the bottom of the environmental

chamber adjusting to 37°C. The chamber was filled with the enzyme solution (0.5 mg enzyme protein/ml of the buffer) and the control strips were immersed in the appropriate buffer (phosphate, pH 7.0 and acetate, pH 4.0).

Results and Discussion

During the aging of beef, postmortem muscle passed through two phases with respect to texture. The process of rigor or muscle extensibility is the first phase and accomplished by 2 days postmortem. Muscle fiber surface does not show the textural changes such as decrease in shear force, decrease in break elongation or breaking strength, except that of muscle fiber shortening. The second phase is the resolution of rigor when the muscle becomes extensible again and it is accomplished 5 or 6 days postmortem. During this phase transverse breaks occur across muscle fibers, sarcomere length is increased and break elongation increase to about 2/3 of the pre-rigor muscle. Considerable degradation of the Z-lines, disappearance of M-line and partial disintegration of I-band, occur at the myofibrillar level. Tenderness of meat is associated with breakdown of sarcolemma and endomysium and increase in sarcomere length. Altough some works disregard proteolysis as contributing factor to postmortem tenderization (Goll *et al.*, 1970), evidence is filing up that proteases present in muscle tissues or macrophages lyse the myofibrillar and connective tissue membranes at both neutral and acidic pH (Jannoff, 1972; Venugopal, 1970; Eino & Stanley, 1973). Proteases and other hydrolases that break biologic macromolecules are present in blood leukocyte lysosomes and these hydrolases have been shown to degrade *in vitro* some isolated myofibrillar proteins (Kim, 1974). Porcine leukocyte lysosmal enzymes were used in the present study because porcine leukocytes are more easily isolated than bovine leukocytes from the respective blood. Proteolytic capacity of the lysosomal enzymes from both sources in quite similar. Transmission electron microscopic studies on fresh bovine *psoas* muscle strips and muscle strips treated with leukocyte lysosomal enzymes at different pH, temperature and time periods reveal that myofibrillar components are degraded by these enzymes. Transmission electron micrograph of fresh bovine *psoas* muscle fixed very shortly after postmortem is shown in Fig. la. The typical ultrastructural features of a straited muscle such as A, I and Z-bands are evident. Sarcomere length averages about 1.81μ.

Micrograph (1b) illustrates the changes that had occurred in the muscle strip when incubated in phoshpate buffer, pH 7.0 for 12 hours at 37°C. The sarcomere length is reduced to 1.36μ but the Z-lines are intact. Treatment with enzyme at pH 7.0 for 12 hours removed the Z-line completely (Fig. 1c) The sarcomere lengths are longer than control (1.5μ). M-line material is degraded. I-band area appears clear. Striatations in A-band area suggests that actomyosin is not greatly affected by these porteolytic enzymes.

Micrograph 1d depicts the muscle strip incubated for 24 hours in phosphate buffer at pH 7.0 and at 37°C. The sarcomere length appears to be reduced to 1.17μ, which could be due to the muscle strip going to rigor. Fig. 1e is a micrograph of enzyme treated muscle at 37°C for 24 hours and it shows complete degradation of Z-line materials.

Micrographs 2e and 2f depict the control and enzyme treated muscle strips in phosphate buffer, pH 7.0, but at 4°C and 36 hours. The sarcomere measures about 1.51μ. The intact Z, M-line and H-zone appear similar to the control strip incubated at 37°C for 12 hours. Enzyme treated samples (2f) shows Z-line break down but the disintergration is not as pronounced as at 37°C for 12 hours. Micrographs 3a and 3b depict the control and enzyme treated muscles at pH 7.0 at 4°C for 7 days. In the control sample, the sarcomere measures 1.35μ in length and M and Z-lines were intact. But Z and M-lines had completely disintegrated in the enzyme treated sample (3b). The activity of lysosomal enzymes on muscle strips in acetate buffer pH 4.0 at 37°C and 4°C is shown in Fig. 2a, b, c, d, and Fig. 3c, d, e, f. Incubation of muscle strips in the acetate buffer, pH4.0, without the enzyme treatment disintegrates to M-line material. The lysosomal enzymes were not very active in disintegrating the Z-line at this acidic pH.

Micrograph 2a and 2b depict the control and enzyme treated muscle at 37°C for 12 hours at pH 4.0.

M-lines have completely disintegrated even in the untreated muscle. The sarcomere measures 1.88μ in length. A partial breakup of protein material at the junction of A-I band is noticed. The enzyme treated muscle has not undergo any appreciable change at the Z-line area but the I-band area appears to have disintegrated. Micrographs 2c and 2d depict the effects of enzyme treatment for 24 hours at pH 4.0. These micrographs appear to be similar to micrographs 2a and 2b. Z-line appears to be stable even after 24 hour incubation. Micrographs 3c and 3d depict the effect of enzymes on the muscle strips at 4°C for 36 hours. In the control muscle, Z-lines appear to be stable but M-lines and myofilaments have disintegrated. Sarcomere measures about 1.89μ in length. Enzyme treated muscle has undergo partial disintegration at the Z-line area and the sarcomere extended in length to 2.11μ. Muscle strips subjected to lysosomal enzyme preparation at pH 4.0 and at 4°C for 7 days appear to have undergo complete degradation of myofibrillar structure. Both treated and untreated muscle strips have lost the characteristic striated ultrastructure.

As indicated above, muscle incubated with leukocyte lysosomal enzymes at pH7.0 produced distinguishable degradation of Z-line, M-line and H-zone at both 37 and 4°C. These observations suggest that neutral proteolytic enzymes present in leukocyte lysosomes have very high activities for degrading muscle structures even at refrigeration temperature (4°C). At acidic pH (pH 4.0) Z-lines were very stable but M-lines and myofilaments are severely disintegrated at this pH. These results indicate that leukocyte lysosomal enzymes have pH dependent specificity for degrading muscle proteins.

The isometric tension pattern of bovine *psoas* muscle immersed in phosphate buffer, pH 7.0, was compared in Fig. 4. with that of muscle suspended in leukocyte lysosomal enzymes under the same conditions. Maximum tension(55g/cm²) was developed after 2 hours post-mortem in both control and enzyme-treated muscle strips, thereafter a rapid drop to one-half of maximum tension occured for both at 5 hours post-mortem. After 5 hours, decrease of isometric tension in enzyme treated muscle was faster than that

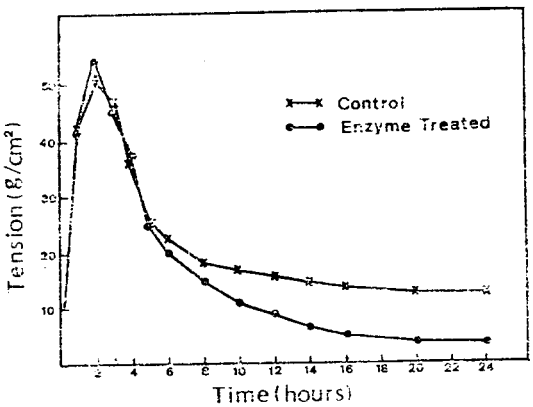

Fig. 4. Effect of leukocyte lysosomal enzymes on post-mortem isometric tension of bovine *psoas* muscle at pH 7.0

The size of muscle strips were 5cm long and 0.25cm² in cross-section. Muscle strips were incubated in 0.5M phosphate buffer(pH 7.0) containing 1mM NaN_3 as antimicrobial agent at 37°C for 24 hours. The enzyme-treated sample contained 0.5mg of enzyme proteins per ml of porcine leukocyte lysosomal enzyme solution but control contained no added enzymes. Isometric tension was measured with an E & M Physiograph. Data are means of four replications.

of control muscle. The tension of enzyme-treated muscle was equal to the initial pre-rigor tension (10g/cm²) after 12 hours but control muscle did not return to this point even after 24 hours. The tension obtained after 12 hours (18g/cm²) was essentially unchanged during the next 12 hours. The more rapid drop of isometric tension in leukocyte lysosomal enzyme-treated muscle compared to control is believed to be due to degradation of the myofibrillar structures as evidenced by results from electron microscopic studies. The isometric tension profiles of muscle immeresed in acetate buffer, pH 4.0 are presented in Fig.5 The tension patterns were different from those of muscle immeresed in buffer, pH 7.0. The tension increased to about 28g/cm² after one-half hour post-mortem and then decreased to 23g/cm² before reaching maximum tension (34g/cm²) 4 hours post-mortem in both control and enzyme-treated muscle. The maximum tension (34g/cm²) of muscle at pH 4.0 was lower than that of muscle at pH 7.0. This is believed to

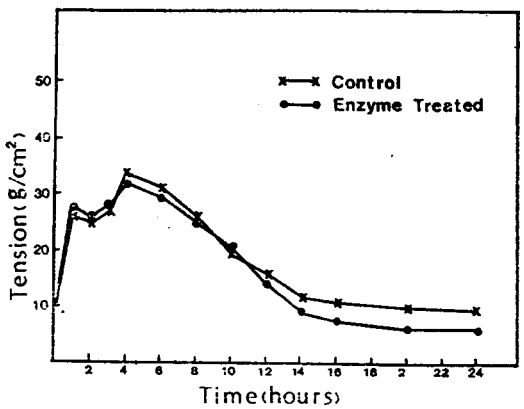

Fig. 5. Effect of leukocyte lysosomal enzymes on post-mortem isometric tension of bovine *psoas* muscle at pH 4.0

The size of muscle strips were 5cm long and 0.25cm² in cross-section. Muscle strips were incubated in 0.1M acetate buffer (pH 4.0) containing 1mM NaN_3 as antimicrobial agent at 37°C for 24 hours. The enzyme-treated sample contained 0.5mg of enzyme proteins per ml of porcine leukocyte lysosomal enzyme solution but control contained no added enzymes. Isometric tension was measured with an E & M Physiograph. Data are means of four replications.

be due to disordering of myofilaments at the lower pH resulting in decreased muscle contractability. The effect of added leukocyte lysosomal enzymes on isometric tension at pH 4.0 was not significantly different from that of control although the tension of enzyme-treated sample was less than that of the control after 12 hours. These results were confirmed by data from electron microscopy as discussed earlier.

要 約

돼지白血球 lysosomal효소를 여러 pH(7.0, 4.0), 溫度 및 處理時間(37°C에서 12, 24時間. 4°C에서 36, 168時間)으로 處理한 牛의 腰筋纖維와 超微細的變化를 TEM으로 관찰한 바 pH 7.0에서는 처리溫度와 時間에 관계없이 Z-line, M-line, H-zone등의 分解를 나타내었으나, pH 4.0에서는 myofilament만이 顯著한 分解를 나타내었을뿐, Z-line은 正常的이어서 分解作用이 없음을 보였다.

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Fig.1, 2 and 3. Transmission Electron Micrographs of Bovine Psoas Muscle

1a (X14, 300): Pre-rigor muscle

1b (X22, 230): Control muscle incubated without leukocyte lysosomal enzymes in phosphate buffer(pH 7.0) at 37°C for 12 hours

1c (X15, 760): Enzyme-treated muscle incubated with leukocyte lysosomal enzymes in phosphate buffer(pH 7.0) at 37°C for 12 hours.

1d (X25, 730): Control muscle incubated without leukocyte lysosomal enzymes in phosphate buffer(pH 7.0) at 37°C for 24 hours.

1e (X44, 120): Enzyme-treated muscle incubated with leukocyte lysosomal enzymes in phosphate buffer(pH 7.0) at 37°C for 24 hours.

2a (X16, 050): Control muscle incubated without leukocyte lysosomal enzymes in acetate buffer(pH 4.0) at 37°C for 12 hours.

2b (X14, 530): Enzyme-treated muscle incubated with leukocyte lysosomal enzymes in acetate buffer(pH 4.0) at 37°C for 12 hours.

2c (X16, 400): Control muscle incubated without leukocyte lysosomal enzymes in acetate buffer(pH 4.0) at 37°C for 24 hours.

2d (X12, 410): Enzyme-treated muscle incubated with leukocyte lysosomal enzymes in acetate buffer(pH 4.0) at 37°C for 24 hours.

2e (X16, 500): Control muscle incubated without leukocyte lysosomal enzymes in phosphate buffer(pH 7.0) at 4°C for 36 hours.

2f (X17, 890): Enzyme-treated muscle incubated with leukocyte lysosomal enzymes in phosphate buffer(pH 7.0) at 4°C for 36 hours.

3a (X20, 340): Control muscle incubated without leukocyte lysosomal enzymes in phosphate buffer(pH 7.0) at 4°C for 168 hours.

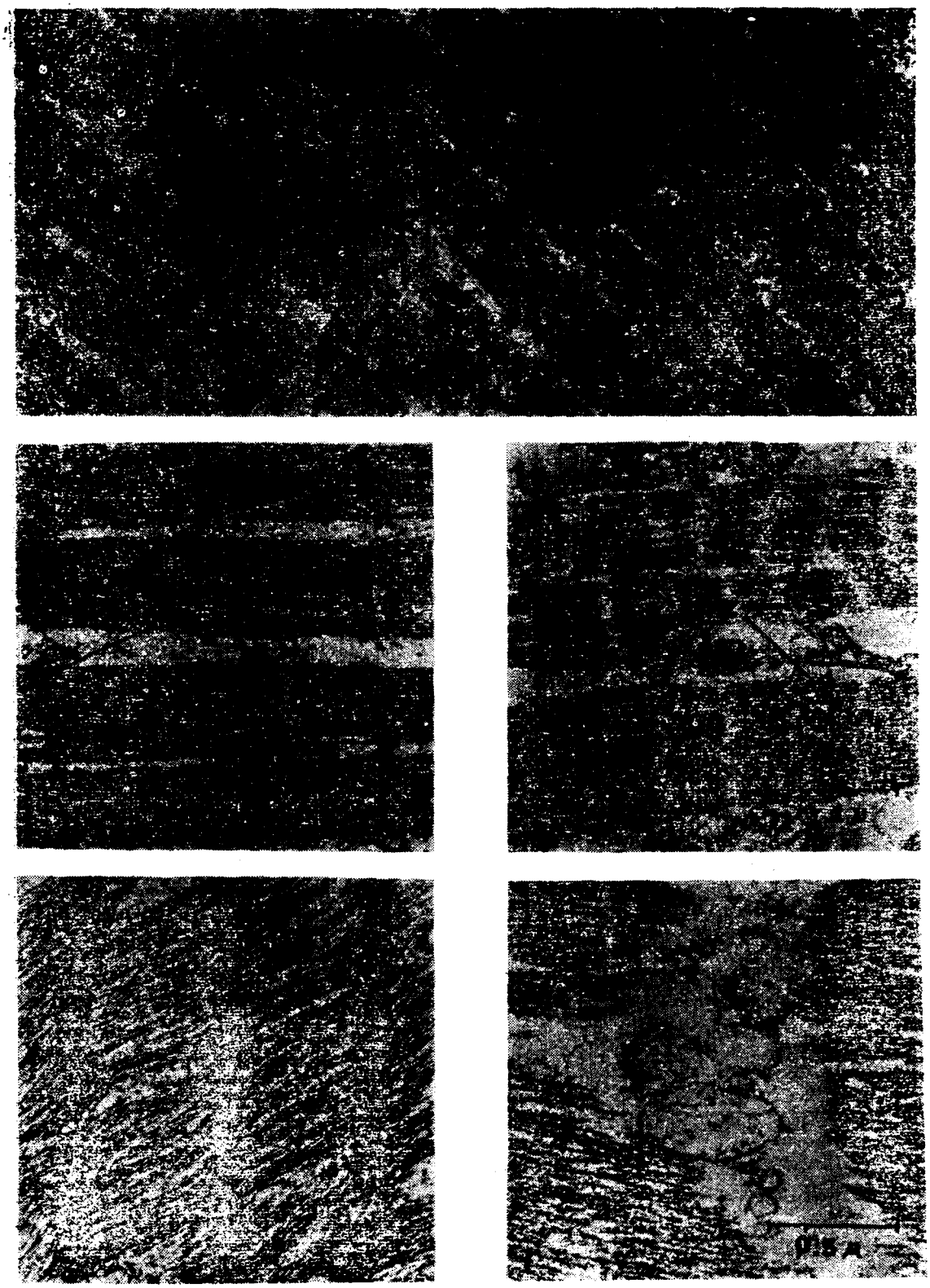
3b (X31, 920): Enzyme-treated muscle incubated with leukocyte lysosomal enzymes in phosphate buffer(pH 7.0) at 4°C for 168 hours.

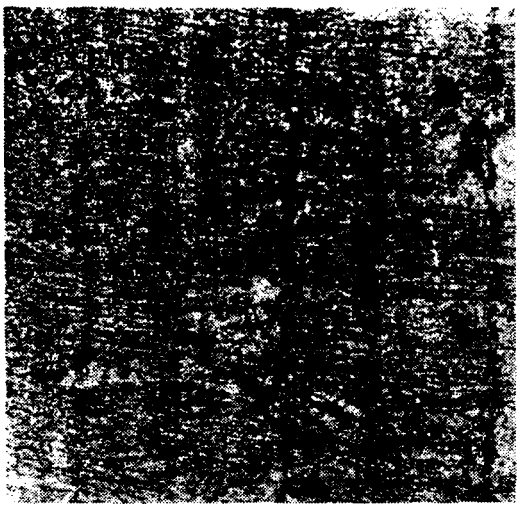
3c (X13, 460): Control muscle incubated without leukocyte lysosomal enzymes in acetate buffer(pH 4.0) at 4°C for 36 hours.

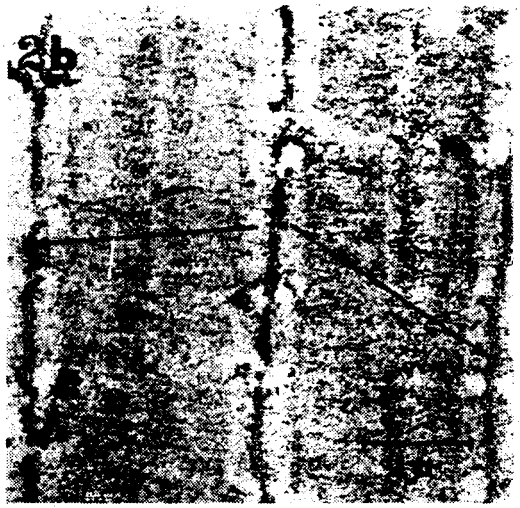
3d (X15, 930): Enzyme-treated muscle incubated with leukocyte lysosomal enzymes in acetate buffer(pH 4.0) at 4°C for 36 hours.

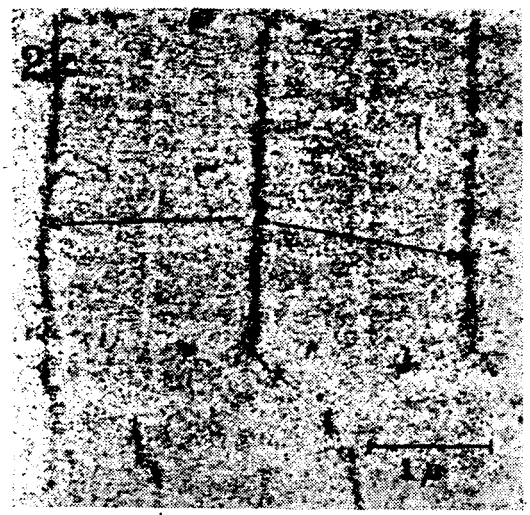
3e (X11, 480): Control muscle incubated without leukocyte lysosomal enzymes in acetate buffer(pH 4.0) at 4°C for 168 hours.

3f (X15, 890): Enzyme-treated muscle incubated with leukocyte lysosomal enzymes in acetate buffer(pH 4.0) at 4°C for 168 hours






2b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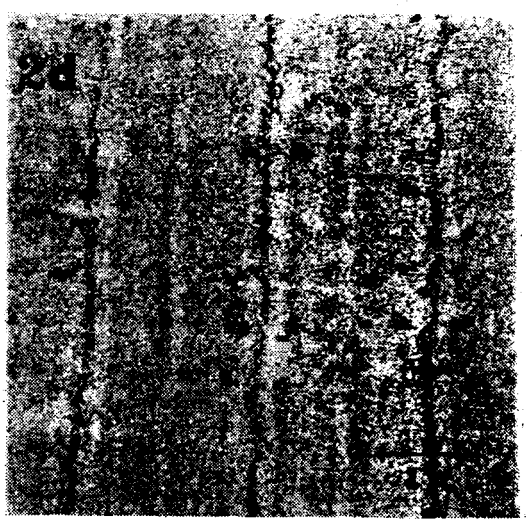
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