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Bovine Muscle Cathepsin D: Purification and Proteolytic Activity on Muscle Proteins

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BOVINE MUSCLE CATHEPSIN D: PURIFICATION AND PROTEOLYTIC ACTIVITY ON MUSCLE PROTEINS

by

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Paul Hwaluen Fan

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ABSTRACT

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by

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An affinity column for cathepsin D was prepared making use of the strong affinity of pepstatin for cathepsin D. Pepstatin is an N-acylated pentapeptide from Actinomycetes with the following structure: isovaleryl-L-valyl-L-valyl-4-amino-3-hydroxy-6-methylheptanoyl-L-alanyl-4-amino-3-hydroxy-6-methyl heptanoic acid. A relatively rapid and efficient method for cathepsin D purification has been developed; Steps include homogenization, ammonium sulfate fractionation, and chromatography on pepstatin-Sepharose column. The final preparation has a specific activity of 38 units/mg. and shows a single protein band on polyacrylamide gel electrophoresis in sodium dodecyl sulfate corresponding to a subunit molecular weight of 42,000. Polyacrylamide gel electrophoresis studies did not reveal any impurities. The proteolytic activity of isolated cathepsin D on bovine myofibrils and myosin was

examined at pH 3.80, 37 °C. The heavy chains of myosin, as well as other smaller regulatory proteins of the myofibrils were degraded. Actin was degraded less rapidly than myosin heavy chain. Degradation became more extensive when the substrate-enzyme incubation time was increased.

(86 pages)

INTRODUCTION

Lysosomes contain several acid hydrolases within the lysosomal membrane. Under certain conditions such as low pH, physical disruption and treatment with detergents the lysosomal membrane is rendered liable. This will release the lysosomal enzymes into the surrounding milieu. Lysosomal enzymes have been identified in many animal tissues. The studies of De Duve and Wattiaux (34) have shown that the lysosomal system played a key role in intracellular protein turnover and resorption of articular and connective tissue, both in physiological remodelling and during physiological damage (42, 124). In 1974, Dr. Christian De Duve was awarded a share of the Nobel Prize for medicine for work on lysosomes which reflects the importance of the lysosome concept in cell biology.

Some 40% of the total body protein is muscle protein and 60% of that is myofibrillar protein (165). Under physiological conditions it is thought that myofibrillar proteins constitute a large and highly mobile protein reservoir. Myopathies and advanced starvation are typified by the disruption and disappearance of the orderly array of these myofibrillar proteins. Few proteinases, other than those associated with lysosomes have been identified in skeletal muscle. An alkaline proteinase has been isolated that appears to be bound to the myofibrils (93). Katunuma et al. (67) have isolated an enzyme that selectively degraded pyridoxine-dependent enzymes. Also,

Dayton et al. (31) have isolated a Ca^{2+}-activated neutral proteinase which has been reported to be the same proteinase as described by Huston and Krebs (63) and Busch et al. (18). Of these, only the Ca^{2+}-activated proteinase has been purified and shown to have proteolytic activity on isolated myofibrillar proteins. The Ca^{2+}-activated proteinase, a sarcoplasmic enzyme, removed Z lines and partly degraded M lines, and can degrade troponin, tropomyosin and C-protein (18, 31, 32). It cannot, however, degrade myosin or actin, and Dayton et al. (32) suggested that the enzyme might have a role in the disassembly of intact myofibrils during myofibrillar protein turnover. In 1977, Schwartz and Bird (126) isolated cathepsin D from rat liver and skeletal muscle and showed that the purified enzyme could degrade myosin as demonstrated by sodium dodecyl sulfate polyacrylamide gel electrophoresis.

Proteolytic enzymes are known to increase the tenderness of meat when properly used, and the tenderizing effect is undoubtedly dependent upon the ability of the enzyme to degrade the muscle proteins (66). Proteolytic enzymes of plant origins such as papain, bromelin, and ficin have been used extensively in commercial meat processing (18, 19, 20, 23, 51, 66, 129, 147, 159).

Among the lysosomal enzymes, cathepsin D (E.C.3.4.23.5.) has excited considerable interest because it is a major proteolytic enzyme of the lysosome as reported by De Duve and Wattiaux (34) and Dingle (35) which was primarily responsible for the extracellular and intracellular degradation

of the organic matrix of skeletal tissues.

The integrity of the lysosomal membrane and the activity of cathepsin D in postmortem muscle is of interest, since the acid hydrolase present in lysosomes could be responsible, at least partly, for the postmortem breakdown of proteins and other components in muscle, which is concomitant with tenderization of meat during conditioning (37).

In order to study the effects of cathepsin D on in vitro muscle protein turnover, as well as postmortem meat quality, it is necessary to isolate cathepsin D, the main acid proteinase in lysosome (8, 11, 34). The bovine skeletal muscle was chosen not only because cathepsin D from this tissue has not been studied before, but also because its endogenous enzyme activity against muscle protein is of interest. Therefore, the objective of this work is to (A) isolate lysosomal cathepsin D from bovine skeletal muscle tissue, (B) prepare myofibrils and myosin muscle proteins, and (C) determine the proteolytic activity, if any, of the isolated enzyme on these proteins.

REVIEW OF LITERATURE

General Background

Cathepsins have been studied in many animal tissues, although none have been fully characterized (36). Those of the spleen were studied by Anson (3), using his hemoglobin assay to follow the activity (2). At present, five types of catheptic activity have been described, namely: cathepsin A, B, C, D, and E. Cathepsin A, B, and C have been characterized by Fruton et al. (45) according to their ability to degrade the synthetic substrates α-N-benzylcarbonyl-L-glutamyl-L-tyrosine, α-N-benzyl-L-argininamide, and α-N-acetyl-L-tyrosinamide, respectively. In 1960, Press et al. (114) isolated a proteolytic enzyme from spleen which accounted for two thirds of the proteolytic activity of a mince of that tissue when the hemoglobin assay of Anson (2) was used to follow activity. It has been classified as cathepsin D since it was inactive against the substrates hydrolyzed by cathepsins A, B, and C. It was concluded by these investigators that cathepsin D was probably identical with the proteinase partially purified by Anson (3). Another proteinase, cathepsin E, differing from cathepsin D in pH optimum and charge at pH 8.2 (79), has been isolated from rabbit bone marrow (79). It is also inactive against the synthetic substrates for cathepsins A, B, or C (79).

Among the lysosomal enzymes, cathepsin D has been reported to be the major proteolytic enzyme by Dingle (35). The

concentration of this enzyme was greatly elevated in tissues undergoing rapid resorption such as the tail of metamorphosing tadpoles (153), and the involuting mammalian uterus (161). It has also been suggested that cathepsin D plays an important role in the turnover of extracellular and intracellular proteins (33, 35), and in the pathological processes of tissue breakdown such as in osteoarthritis (124, 156, 162) and muscular dystrophy (155).

Properties of Meat

Tenderness is the most desired eating quality of meat as served at the dining room table. The factors influencing tenderness in meat are not completely understood. Meat fibers and connective tissue are meat elements which influence tenderness. Because of variation in content of these elements, tenderness varies between different sections of the same muscle, between different muscles in the carcass, and between similar muscles from different carcasses (28, 56, 57, 94, 120, 130).

To understand the properties of meat which makes it tough, it is essential to consider the primary components of meat. Lean muscle consists of approximately 20% protein, 60-70% water, 9% fat, and 1% ash (106, 108). These proportions will change as the animal is fattened, which will result in a decrease in the percentage of protein and water and a

proportional increase in fat (119). Although water contributes to many of the characteristic properties of meat, it does not influence the tenderness or toughness of meat as compared with the other meat components (106).

Fat is located within the muscle cell, between muscle cells, between muscle bundles and covering the whole muscle. The muscle cell contains about 1-2% fat (36), while the whole muscle may contain up to approximately 10% fat (not including the external fat cover). It is known that fat is not a tough tissue and therefore it does not contribute to meat toughness.

Protein comprises about 20% of the meat. Meat proteins are typically classified into three groups: sarcoplasmic, myofibrillar (contractile), and connective tissue proteins.

Sarcoplasmic proteins comprises the fluid, viscous plasma of the cell. These proteins are relatively small and are soluble in water, and consequently do not influence the toughness of meat (78, 119). The remaining two protein classes are the principal contributors to meat toughness, yet these two proteins have some basic differences. Myofibrillar (contractile) proteins are structural proteins within the muscle cell that form the muscle filaments. These filaments run for varying distances in muscles and are responsible for contraction in living muscle. In muscles that have relatively small amounts of connective tissue, such as rib and loin muscles (120), the myofibrillar proteins are greater contributors to meat toughness (119).

The connective tissue proteins are located primarily

outside the muscle cell to support and maintain the register and integrity of the muscle cells and muscle bundles (121, 130). These proteins transmitted muscle contraction to the skeleton. Thus, connective tissue must be strong and inelastic. Muscles of locomotion or continual activity, such as leg, neck or heart muscles, have considerably greater amounts of connective tissue than support muscles such as the rib and loin muscles (120). As an animal matures, connective tissue proteins becomes even more inextensile even though the amount of connective tissue remains constant (11, 20, 58). On the other hand, myofibrillar protein toughness does not significantly change as the animal grows older (20, 78, 120).

Collagen is the major protein in connective tissue and this protein is greatly responsible for meat toughness because it occurs in great amounts in active muscles and it will form crosslinkages as a mature animal grows older, which will make it tougher (20, 58, 83, 119).

Myofibrils

The intracellular proteins of skeletal muscle cells consist of the water-soluble proteins of the sarcoplasm, which make up about 20 to 25 percent of the total muscle proteins, and the water-insoluble filamentous proteins of the myofibrils. The myofibril is the smallest integral con-

tractile unit of the muscle, consisting of a highly organized complex of contractile proteins, i.e., myosin and actin, as well as regulatory and structural components (41, 78). The chemical and structural changes occurring in the postmortem aging of muscle are directly related to the degradation and alterations of myofibrillar structure of the muscle (84, 103, 147). These changes at the myofibrillar level include: 1) a drop of sarcoplasmic reticulum ATPase activity, 2) a drop of pH from 7.1 to 5.2 followed by; 3) subsequent disappearance of Z lines (29, 136, 160), 4) fragmentation of myofibrillar structure into smaller components with the appearance of a 30,000 dalton fragment (103, 104), and 5) an increase in the dissociation of the actomyosin complex (47, 50).

Many of these postmortem changes in myofibrillar proteins have generally been assumed to arise from the release and activity of endogenous muscle proteases active at postmortem pH (37). Dutson et al. (38) reported that the increased activity of lysosomal proteases and the physical disruption of myofibrils were observed in postmortem electrically stimulated muscles. Since skeletal muscle cells contain lysosomes, several groups have investigated the effect of the lysosomal proteinases, such as cathepsins, on myofibrils. Eino and Stanley (39, 40) observed that a preparation of muscle cathepsin D incubated with myofibrils under postmortem pH (3.8) conditions produced structural changes similar to those observed during postmortem aging of muscles. Robbin and Cohen (117) treated bovine myofibrils at pH 5.3 with an extract of

bovine spleen, which contained a high concentration of cathepsin D and also microscopically observed selective degradation of the Z disk region. Later studies of the same authors (117) demonstrated that cathepsin D caused a degradation of Z disks of the bovine myofibrils and had a relatively selective action on the myofibrillar proteins. The heavy chain of myosin (200,000 daltons) was degraded to fragments of about 170,000, 150,000, and 80,000 daltons at 25 °C. Degradation became more extensive at 37 °C. In treatment of the muscle tissue with spleen extract, these authors further observed that both the sarcolemma and stroma of muscle appeared to be degraded. Recently, Schwartz and Bird (126) reported that rat liver and muscle cathepsin D degraded the heavy chains of isolated myosin to fragments of about 175,000 daltons to 150,000 daltons and then subsequently to fragments of 100,000 daltons which appeared to be resistant to further degradation. Arakawa et al. (6) reported that a catheptic enzyme from rabbit muscle with properties similar to cathepsin D degraded the heavy chains of myosin present in myofibrils to fragments of 150,000 daltons and 70,000 daltons.

Several publications (31, 32, 103) have shown that a Ca^{2+}-activated protease designated as calcium activated factor (CAF), can degrade myofibrillar proteins. This protease, CAF, possessed optimum proteolytic activity at pH 7.5 and activity decreased rapidly below pH 6.5 or above 8.0 (128). Further, CAF was shown to remove Z lines from

intact muscle myofibrils, degrade troponin T, tropomyosin and C-protein components of muscle (31, 32). When the troponin complex was incubated with CAF, troponin T was degraded to a 30,000 dalton residue (104). These observations thus suggested that CAF might play a significant role in the turnover of muscle tissue, although its role in postmortem proteolysis, where pH is low, has not been demonstrated (32).

In view of the above observations, it seemed that for a clear understanding of the role of catheptic enzymes on the postmortem conditioning of muscles, selective analytical techniques which examine integral structure changes must be investigated. The study of the myofibrillar system advanced dramatically with the development of the technique of polyacrylamide gel electrophoresis in sodium dodecyl sulfate as a means of analyzing the molecular weight, composition and purity of these proteins (86, 127, 128).At present, the standard sodium dodecyl sulfate-polyacrylamide gel electrophoresis procedures of Weber and Osborn (154) used in the analysis of myofibrillar proteins are a compromise in which adequate entry and resolution of the myosin heavy chain is gained with loss in resolution of the smaller molecular weight components. Conversely, when adequate separation of the smaller protein subunits is obtained, incomplete entry of myosin heavy chain into the gel usually results (113). Thus, the need for an SDS-polyacrylamide gel electrophoresis method which would adequately resolve myosin, actin, and the regulatory proteins led Porzio and Pearson (113) to

modify the standard procedures of Weber and Osborn (154) to obtain improved separation and resolution of the myofibrillar components with a single gel system.

After an evaluation of the effects of gel concentration, running current, buffer concentration and pH, Porzio and Pearson selected a system which gave optimum resolution of peptides over the widest possible size range. The running gel consisted of 10% acrylamide cross-linked with 0.01% bis-acrylamide (100:1) incorporating 400 mM Tris-glycine buffer (pH 8.80), 5% glycerol, 0.1 mM EDTA and 0.1% SDS. Using current as a dependent variable, SDS-polyacrylamide gel electrophoresis with this system was best achieved at 0.75-1.0 mA per gel at ambient temperature with the corresponding times 4-6 hours. Higher currents result in distortion of protein bands due to localized heating within the gel. The incorporation of 5% glycerol in the gel reduced the extent of band distortion when migration proceeded at 1.0-1.25 mA per gel. Lower running currents were employed but without significant improvement in resolution.

Physico-Chemical Properties of Agarose Gel

Agar agar has been isolated from various species of red sea weed and consists of two principal polysaccharide components: (a) agaropectin, which consists of 6% charged sulphate groups, pyruvic acid and glucuronic acid, and (b) agarose, which is a neutral linear polysaccharide consisting of alter-

nating D-galactose and 3,6-anhydro-L-galactose units (86). These polysaccharides have been resolved by fractional precipitation with polyethyleneglycol (121) or by precipitation of the agaropectin with cetylpyridium chloride (59).

Agarose is freely soluble in boiling water and forms an insoluble gel on cooling below 40°C. even at concentrations as low as 0.4%. In contrast to dextran and polyacrylamide gels, the polymer chains are held together by hydrogen bonds and not by covalent linkages (86). This means that agarose gel is less stable chemically than the covalently crosslinked dextran or polyacrylamide gels. Thus, agarose gels are less stable to extremes of pH, and exposure to media with pH values outside the range of 4-9 should be avoided (24, 112). Reagents which disrupt hydrogen bonds can decrease the mechanical stability of the gels (44, 54) if the agarose content is low (2%) although, in general, moderate concentrations of urea or guanidine-HCl are well tolerated. Furthermore, the gels are not disrupted by organic solvents such as ethanol or acetone, whereas dextran and polyacrylamide gels will shrink completely when treated with acetone (24). However, it should be noted that agarose is a thermally reversible gel and on heating the gels lose stability and eventually melt. Thus, those gels must be kept at low ambient temperatures, although not frozen, since freezing also results in irreversible structural disruption (112). So in practical terms, heat sterilization is impossible and the gels must be stored in the wet state since they can not be dried and reswollen. However,

the derivatized agarose are often more stable to heat or solvents than the parent gels (24, 25).

The main producers of agarose are Pharmacia (Uppsala, Sweden), under the trade-name Sepharose, and Bio-Rad Laboratory (Richmond, Calif., U. S. A.), under the trade-name Bio-Gel A. Sepharose is an agarose gel with spherical particles, and is sold in the swollen state, suspended in water containing 0.02% of sodium azide as a bacteriostatic agent. AH-Sepharose 4B has a free amino group at the end of 6-carbon spacer arms for coupling ligands containing free carboxyl groups. It is formed by covalent linkage of 1,6-diaminohexane to Sepharose 4B using the cyanogen bromide activation method (7, 26, 89). Treatment of these gels with cyanogen bromide during manufacturing increases their stability in the presence of organic solvents, thus expanding the possibilities for derivatization (24, 25, 105).

Pepstatin and Mode of Inhibition

Pepstatin is a low molecular weight, potent inhibitor specific for acid proteases (4, 5, 12, 23, 76, 96), discovered by Umezawa and his co-workers (150) in the culture filtrates of various species of Actinomycetes. It has been shown to inhibit nearly all acid proteases including porcine pepsin, renin, cathepsin D (5), bovine chymosin (143), human pepsin and gastricsin, and several acid proteases of microbial origin (4).

The unusual potency of pepstatin toward acid proteases was indicated by its K_i which was reported by Kunimoto et al. (77) to be about 1×10^{-10} M for porcine pepsin. Due to this remarkable property, pepstatin has been widely used as a research tool in the studies of enzyme mechanism (122), biological functions (12), and in affinity chromatography (23, 100). It also has been tested as a therapeutic agent for the experimental control of gastric ulcer (150), carrageenin edema (150), and hypertension (81, 96).

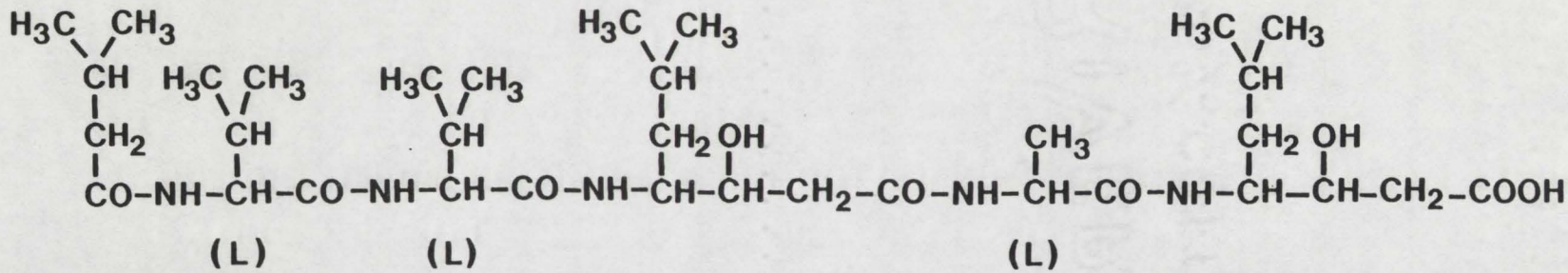
Affinity chromatography with immobilized pepstatin has been successfully employed in the preparation of pure and undegraded cathepsin D from rat liver (82), bovine brain (16, 157), thymus (74) and spleen (52, 149), but the mode of inhibition is still not known.

The chemical structure of pepstatin was determined by Morishima et al. (98) to be a pentapeptide which contains two residues of an unusual amino acid, 4-amino-3-hydroxy-6-methylheptanoic acid (statine is the name proposed for this unusual amino acid). The complete structure of pepstatin is iso-valeryl-L-valyl-4-amino-3-hydroxy-6-methylheptanoyl-L-alanyl-4-amino-3-hydroxy-6-methylheptanoic acid (Figure 1). It has been shown by Kunimoto et al. (76) that esterification of the pepstatin carboxyl terminus has no effect on its inhibitory activity but that acetylation of the hydroxyl group of each statyl residue drastically reduces the inhibition by pepstatin.

It is known from the use of two types of active-site directed irreversible inhibitors, the diazo inactivators (14, 69, 88) and the epoxide inactivators (21, 55, 145), that pepsin contains two aspartyl residues which apparently are essential for enzymic catalysis (14, 21, 55, 145). These same inactivators were also reported to react with and inactivate many other acid proteases in a similar manner (69, 70, 72, 131, 133, 142), including cathepsin D. The implication of these studies is that the acid proteases possess similar active center structures and a common mechanism of catalysis (90). Based on these assumptions, Marciniszyn et al. (90) suggested a common mode of inhibition by pepstatin to acid proteases. They found that although N-acetyl statine was a weaker inhibitor than pepstatin, it was nevertheless a relatively strong inhibitor of acid proteases as compared with its structural analog N-acetyl-leucine. The inhibitory constant of N-acetyl-statine (1.2×10^{-4} M) was almost 600-fold less than that of N-acetyl-leucine (7×10^{-2} M) (65). This drastic difference in K_i between these closely related structures (statine and leucine) suggests that N-acetyl-statine is a transition state analog of peptic catalysis (71. 164), and is the source of the inhibitory potency of pepstatin. The strongest support for this proposal was the findings reported by Marciniszyn, Hartsuck, and Tang (90): First, the difference in K_i values between N-acetyl-statine and N-acetyl-leucine, a product of peptic hydrolysis, is similar to that found for a transition state analog of elastase, peptidyl

alanal, and the peptidyl alanol (146). Second, the presence of two binding sites on pepstatin under some conditions suggests that each statine binds to a pepsin molecule (76, 77). Third, the critical importance of the statyl residue in the inhibition was also supported by the findings of Kunimoto and co-workers (76) that acetylation of the hydroxyl groups in pepstatin greatly reduced its inhibitory activity. Fourth, an examination of the transition state for porcine pepsin catalysis, for example, as proposed by Hartsuck and Tang (55) revealed a high degree of structural similarity to a statyl residue. Figure 2 shows such a comparision. It can be seen that in the proposed model of the transition state, both the carbon atom in the carbonyl group and the nitrogen atom in the amide group must be in the tetrahedral configuration as reported by Marciniszyn et al. (90). This is in contrast to the planner configuration in a normal peptide bond. Consequently, the equivalent atoms in statine would be the carbons at positions 2 and 3, which are both in tetrahedral configuration (90).

Subramanian et al. (138) reported that the above "competitiveness" and "transition state hypothesis" were both supported by X-ray crystallographic results of an acid protease from Rhizopus chinensis in which the reactive site of an active center directed reagent, 1,2-epoxy-3-(p-nitrophenoxy)-propane, overlapped with the pepstatin site.



iso-valeryl-L-valyl-L-valyl-4-amino-3-hydroxy-6-methylheptanoyl-L-alanyl-4-amino-3-hydroxy-6-methylheptanoic acid

Figure 1. The structure of pepstatin.

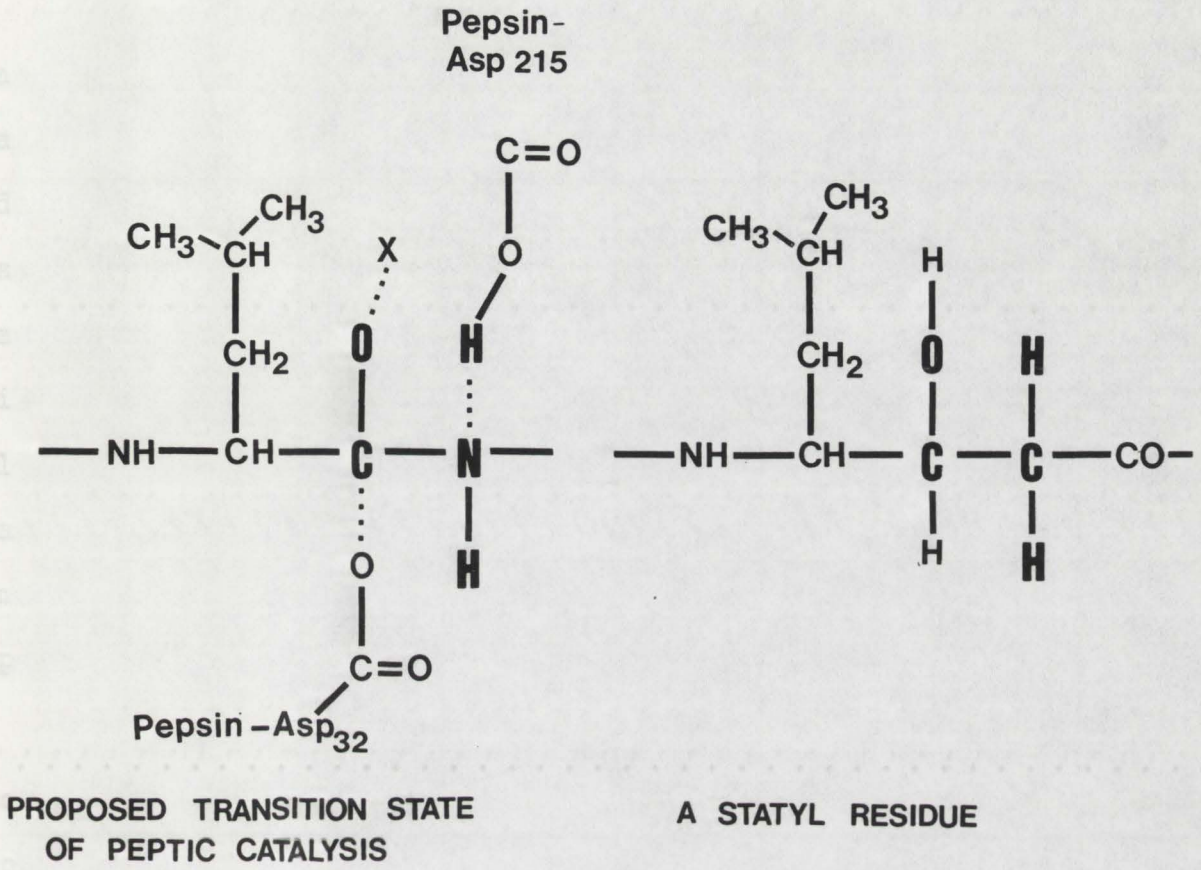


Figure 2. Schematic representation (left) of the proposed transition state in peptic catalysis. Asp_{32} and Asp_{215} are the epoxide- and diazo- reactive aspartyl residues of the enzyme, respectively. X is a postulated electrophile and could be arginine (55). The carbonyl and amide groups of the substrate are shown in bold letters. The N-substituted statine (right) is shown for comparision. The atoms in the statyl residue which are equivalent to those shown in bold print in the proposed transition state, are also shown in bold letters. Adapted from Marciniszyn et al.(90).

Carbodiimide Coupling

Ligands carrying primary aliphatic or aromatic amines and carboxylic acid functions can be coupled to ω-carboxyl-alkyl-Sepharose derivatives and ω-amino-alkyl-Sepharose derivatives respectively by a carbodiimide promoted condensation reaction (60, 61). The mechanism of the carbodiimide assisted reaction is illustrated in Figure 3. The reaction is initiated by the addition of the carboxyl group from the ligand pepstatin across one of the diimide bonds to generate an O-acylisourea. The activated carboxyl group of this intermediate can then react with the AH-Sepharose 4B by nucleophilic attack to yield the acyl-nucleophile product (60, 61).

Several carbodiimides have been used for the synthesis of adsorbents for affinity chromatography (80, 85, 86). Dicyclohexyl-carbodiimide is insoluble in water but has been used very effectively in 80% (v/v) aqueous pyridine for the synthesis of immobilized NAD^+ (80, 86), and for the preparation of an adsorbent for mushroom tyrosinase (53). It was reported that one disadvantage in the use of water-insoluble carbodiimides is that the derived urea is also insoluble in water and consequently must be removed from the gel by extensive washing with organic solvents such as ethanol and butanol (80). However, this problem can be circumvented by using one of the two soluble carbodiimides as reported by Lowe and Dean (85): 1-ethyl-3-(3-dimethylaminopropyl) carbodiimide hydrochloride (EDC) or 1-cyclohexyl-3-(2-morpholinoethyl)

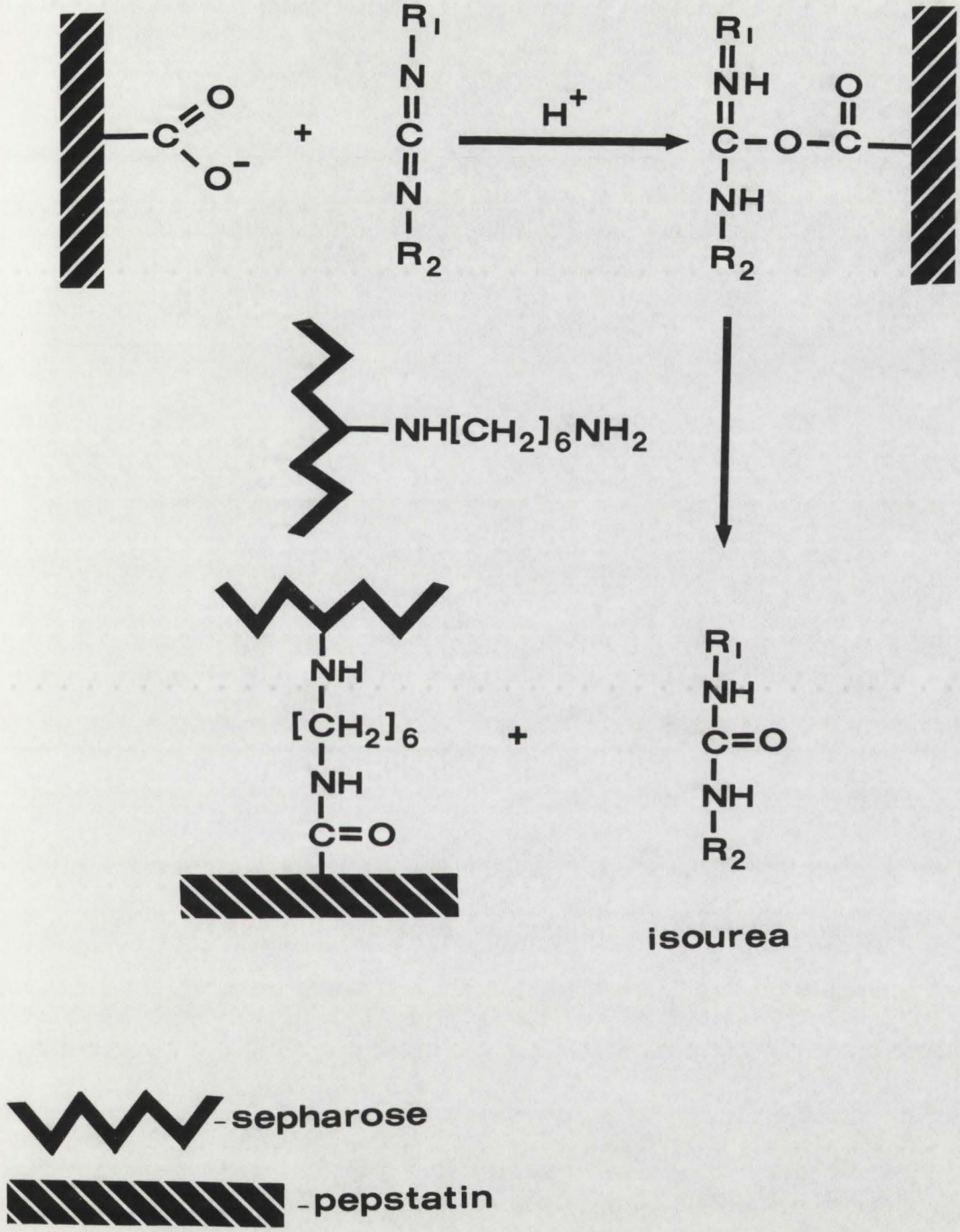


Figure 3. The carbodiimide-promoted reaction.

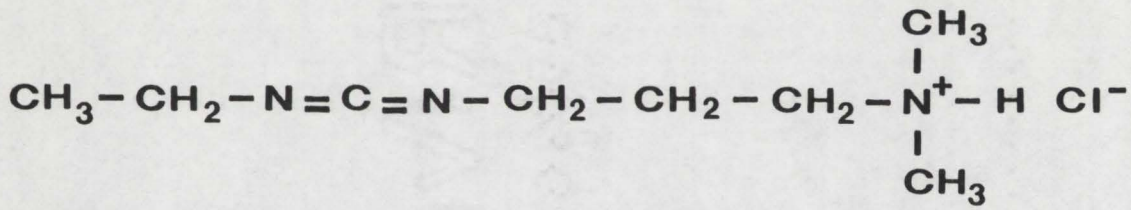


Figure 4. The structure of 1-ethyl-3-(3-dimethylaminopropyl)-carbodiimide hydrochloride.

carbodiimide metho-p-toluene sulphonate (CMC). These two water-soluble carbodiimides are convenient to use since their corresponding urea derivatives are soluble in water and hence, are easily removed from the gel product by washing with water (85).

Cathepsin D Assay

Cathepsin D is routinely assayed by the method of Anson (3) in which denatured hemoglobin is digested under desired experimental conditions. The undigested hemoglobin is precipitated with trichloroacetic acid, and the amount of unprecipitated degradation products, which are produced in proportion to the amount of proteinase present, are estimated with the Folin-phenol reagent. This reagent produces a blue color the intensity of which varies with the proportion of tyrosine and tryptophan present.

The studies of Anson (2, 3) indicated that even when peptidase was present in addition to proteinase, the formation of products not precipitable by trichloroacetic acid was solely due to proteinase alone. It was also reported by Smith and Turk (132) that although the assay with hemoglobin was not specific for cathepsin D, but the total activity of other proteinases present was much lower under these desired experimental conditions.

Purification of Cathepsin D

Although the intracellular proteinase, cathepsin D, has been extensively studied in recent years, it is only understood superficially with regard to its catalytic mechanisms, its structure-function relationship, and its possible function in vivo. Several reasons have contributed to this lack of knowledge of this enzyme. First, this enzyme is present in tissues in relatively low concentrations, particularly in the bovine muscle tissues for reasons unknown. Thus, its isolation is tedious. Cathepsin D has been studied in many animal tissues with several methods. The tissues included rat liver and brain (74, 91), chicken liver (10) and muscle (64, 92), human liver (13) and red blood cells (116), porcine spleen (27), thyroid (75, 131), leukocytes (152) and intestinal mucosa (73), bovine spleen (43, 52, 114, 132, 149), thymus (74), uterus (163) and brain (16, 157), and rabbit muscle (126). Yet the enzyme has not been obtained in large quantity. Second, conflicting reports have appeared on the molecular weight of this enzyme, ranging from 12,000 for bovine uterus cathepsin D (123, 163) to 58,000 for cathepsin D from bovine spleen (43, 52, 114, 132, 149). To date, there has been no agreement on reported molecular weight values for cathepsin D among different tissues. It is not clear whether the cathepsin D of different tissues are active fragments of the same molecules, or actually structurally different isoenzymes. Third, the presence of several isoenzyme

forms in the same tissue has been reported (10, 91, 114, 123), and the number of isoenzymes appears to vary among different tissues. Barrett (10) has described three forms of cathepsin D in chicken and human liver. Twelve forms have been reported in bovine uterus by Sapolsky and Woessner (123), and several forms in rat brain were reported by Marks and Lajtha (91). As yet, no one knows the underlying structural basis of the multiplicity of forms. This confusion concerning the molecular weight and the possible existence of isoenzymes contributes to the difficulties in the structure-function studies.

The first isolated muscle cathepsin D shown to have proteolytic activity upon muscle proteins was from rat skeletal muscle as reported by Schwartz and Bird in 1977 (126). The main steps of the isolation procedure consisted of potassium chloride homogenization, ammonium sulfate fractionation, and dialysis against sodium-acetate buffer. The dialyzed supernatant was then applied to columns of Sephadex G-75 and DEAE-cellulose respectively. The final preparation, in sodium-acetate buffer, had a specific activity of 0.718 units per mg. of protein. The yield was 0.7%. Preparative isoelectric focusing of the purified enzyme resolved three major isoenzymes, and each gave a distinct band when focused in gels (126).

The difficulties in preparing other proteinases and peptidases, such as bovine carboxypeptidases and their zymogens (110, 111, 115, 151), pepsin (2, 15, 45, 46), and trypsin

(2, 15) are well documented. Each of these enzymes must be isolated with due regard for concomitant autolysis or proteolysis by other enzymes present in the tissues. Surprisingly, little attention has been paid to this problem in the isolation of cathepsin D (132). Recent studies of Sapolsky and Woessner (123) and Turk et al. (148) have shown that the enzyme may consist of a single polypeptide chain which undergoes limited cleavage without loss of activity.

It was reported that degradation of the proteolytic enzymes mentioned above can usually be avoided by rapid isolation, or by the addition of inhibitors of other proteinases present (132). In the absence of a detailed knowledge of all the other proteinases present in the tissues, the latter tactic is not possible. Therefore, it is desirable to achieve the isolation as rapidly as possible. The use of affinity chromatography seems to be particularly suited for application to these problems since it is relatively rapid as compared to the classical isolation methods, and a specific substance can be retained on and released from an affinity column in a selective manner (24, 68, 102).

Identification of Cathepsin D

Catheptic activity is a term used to describe acidic proteinase activity found in aqueous extracts of a variety of animal tissues. Cathepsins A, B, and C have been recognized based on their specificity toward N-benzylcarbonyl-α-L-glutamyl-

L-tyrosine, benzyl-L-argininamide, and glycyl-L-phenylalaninamide, respectively (144). Cathepsins D and E, first reported in 1960 and 1962, respectively, have no defined activity towards synthetic peptides (79, 114), since their primary activity is directed against proteins. Cathepsin D is usually assayed at pH 3.8, using hemoglobin as a substrate (2). Iodice et al. (64) found that cathepsin A had a pH optima of 5.4, and showed little or no activity on hemoglobin unless cathepsin D also was present. They concluded that cathepsin A is restricted in its activity to breakdown products of proteins resulting from the prior action of cathepsin D or other proteinases. Similarly, highly purified preparations of cathepsin C are devoid of proteinase activity (101). In fact, Metrione et al. (95) have suggested that cathepsin C be renamed dipeptidyl transferase, in order to more accurately define its action. Barrett and Dingle (12) found that pepstatin inhibited the activity of cathepsin D and cathepsin E, but not cathepsin B. Cathepsin B is a sulfhydryl-dependent enzyme with most proteolytic activity at pH 4 - 5 (101). Thus, cathepsin D preparations derived from a pepstatin affinity column, and assayed at pH 3.8 would have little or no cathepsin B activity. However, a pepstatin affinity column may bind cathepsin E, as well as cathepsin D. Cathepsin E is found in much greater amounts in polymorphonuclear cells than in lymphocytes (79). Bone marrow is thus the best source for cathepsin E, since it is very rich in polymorphonuclear cells (79). Lapresle and Webb (79) were able to clearly separate purified cathepsin D from

cathepsin E by electrophoresis in agar gel at pH 8.2. They showed that cathepsin E was active at pH 2.5, but had virtually no proteolytic activity at pH 3.8. Thus, one may conclude that proteinase activity obtained by use of a pepstatin column and assayed at pH 3.8 with hemoglobin as a substrate may be called cathepsin D, rather than cathepsins A, B, C, or E.

EXPERIMENTAL PROCEDURES

Materials

Fresh bovine skeletal muscle tissues were obtained from the Meat and Physiology Laboratory, Animal Science, Utah State University. AH-Sepharose 4B was purchased from Pharmacia (Piscataway, NJ). Bovine hemoglobin, crystalline bovine serum albumin, pepstatin, 1-ethyl-3(3-dimethylaminopropyl)-carbodiimide, Coomassie brilliant blue R-250, Pyronin Y, dithiothreitol, and molecular weight markers were purchased from Sigma Chemical Co. (St. Louis, MO). Electrophoretic reagents were purchased from Bio-Rad Laboratory (Richmond, CA). Enzyme grade ammonium sulfate was purchased from Schwarz /Mann Inc. (Orangeburg, NY). Ultrafiltration cells and UM-10 membrane were products of Amicon Co. (Lexington, MA). All other reagents were analytical grade. Deionized water was used throughout the experiments.

Myofibril Preparation

Purified myofibrils were prepared from bovine skeletal muscle using the method of Perry et al. (108, 109). The animals were exsanguinated, the muscle was immediately excised, cooled in ice, trimmed of fat and connective tissue, and then cut into small pieces with a razor blade. All

procedures following the excision of the muscle were carried out at 0°C. Volume calculations were based on the original muscle weight unless otherwise specified.

One hundred grams of chilled, minced, freshly excised muscle tissue was homogenized with 5 volumes of borate-KCl solution (0.1 M KCl, 0.039 M borate, 5 mM EDTA, pH 7.1) in a Waring blendor for 1 min. (Figure 5). The connective tissue and residual fat were removed by pouring the homogenate through 4 layers of cheese cloth, and then the filtrate was collected. After centrifuging for 15 min. at 600 x g, the supernatant was discarded. The sediment was resuspended in 5 volumes of borate-KCl solution, homogenized for 2 min., then centrifuged at 600 x g for 15 min. The light-colored upper layer of the sediment, consisting mainly of myofibrils, was removed with the aid of a small amount of borate-KCl solution. The myofibril paste was diluted with 5 volumes of borate-KCl solution, mixed gently, and the coarse material were removed by centrifuging for 3 min. at 400 x g. The sediment was discarded and the suspension was centrifuged again for 15 min. at 600 x g to sediment the myofibrils.

After three further resuspensions and centrifugations to remove sarcoplasmic proteins and granules, the sediment was suspended and centrifuged once more for 3 min. at 400 x g. The sediment was again discarded, and the turbid supernatant was again centrifuged at 600 x g for 15 min. to obtain a concentrated myofibril suspension. The myofibrils were transferred to a flask with a minimum amount of borate-

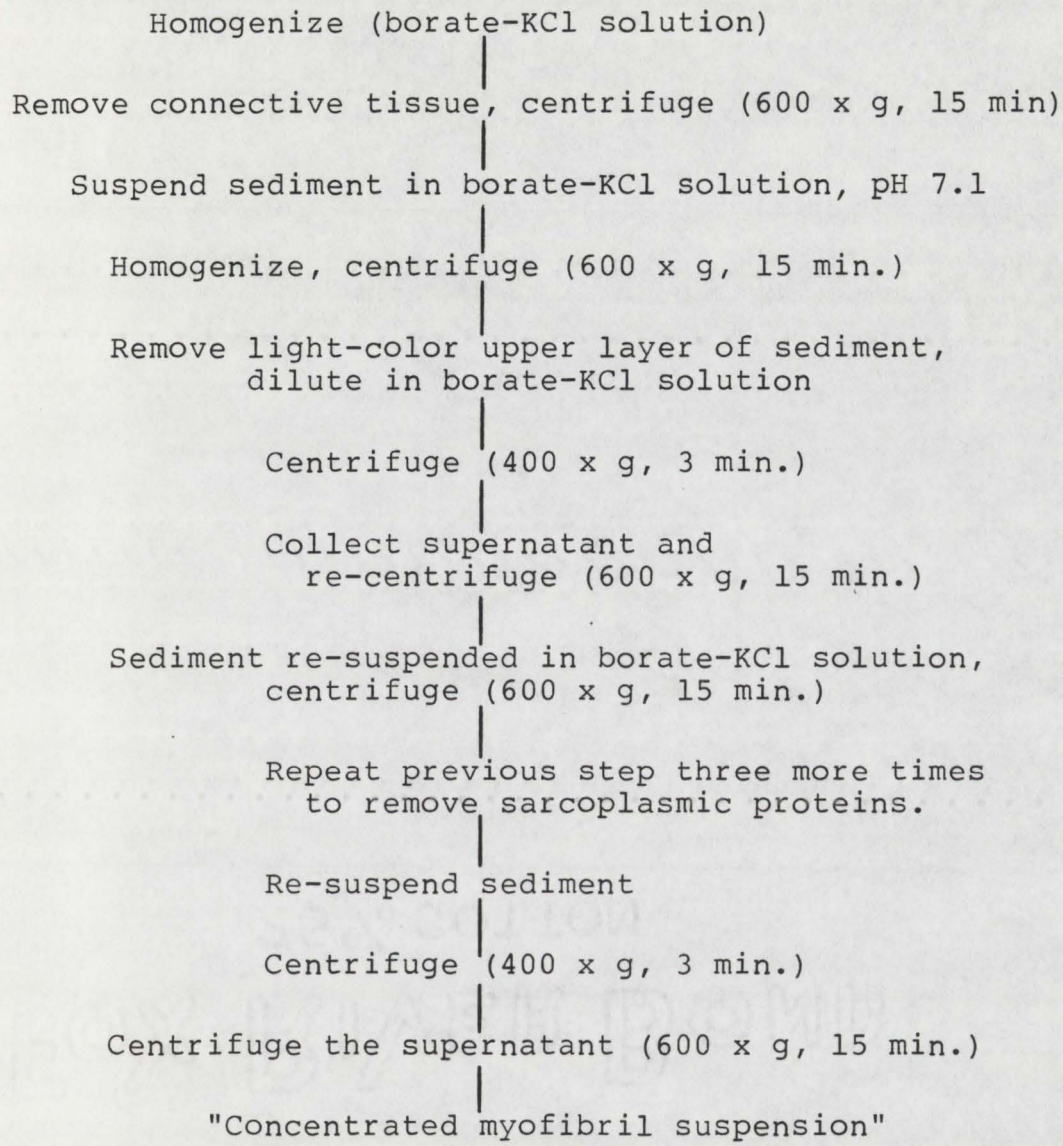


Figure 5. Flow sheet of preparation of myofibrils.

KCl solution, and stored at 0 °C. in the presence of glycerol. This suspension contained 85-90 mg. of protein per ml. and was diluted with 4-5 volumes of borate-KCl solution for enzyme experiments.

Myosin Preparation

Purified myosin was prepared from bovine skeletal muscle as described by Briskey and Fukazawa (166) which was a modification of the methods of Szent-Györgyi (167) and Huxley (168). The animals were exsanguinated, the muscle was immediately excised, trimmed of fat and connective tissue, and then cut into small pieces with a razor blade. All procedures following the excision of the muscles were carried out at 2-3 °C. Volume calculations were based on the original muscle weight unless otherwise specified.

One hundred grams of freshly minced muscle tissue was homogenized with 3.3 volumes of Guba-Straub solution (0.09 M KH_2PO_4, 0.06 M K_2HPO_4, 0.3 M KCl, pH 6.5) in a Waring blendor for 30 seconds. The slurry was then stirred at a moderate speed for 20 min. and dissolved with 13.1 volumes of cold water during moderate stirring. The connective tissue and residual fat were removed by pouring the diluted homogenate through 2 layers of cheese cloth. The filtrate was collected and diluted with 24 volumes of cold water during slow stirring and allowed to stand for 2 hours. The myosin precipitate will

settle to the bottom of the container permitting most of the clear supernatant to be either siphoned off or decanted. The remaining suspension was centrifuged at 1,400 x g for 20 min.

The myosin precipitate was washed once with 0.03 M KCl and dissolved in a minimum amount of Tris-KCl (0.17 M Tris-acetate, 1.3 M KCl, pH 6.8), then the concentration of KCl was diluted to 0.5 M KCl by the addition of cold water. This solution was centrifuged at 31,000 x g for 30 min. to remove the myofibrils. The supernatant was filtered through glass wool to remove lipids, then diluted to 0.27 M KCl by the addition of cold water. The diluted suspension was allowed to stand for 20 min. and then centrifuged at 31,000 x g for 40 min. to remove the actomyosin. The supernatant was diluted to 0.033 M KCl during slow stirring and allowed to stand for 20 min. The myosin precipitate was then collected by centrifugation at 1,400 x g for 20 min.

The myosin precipitate was washed once with 0.03 M KCl and dissolved in a minimum amount of Tris-KCl. The concentration of KCl was then diluted to 0.27 M by the addition of cold water. This solution was allowed to stand for 20 min., then centrifuged at 31,000 x g for 40 min. to remove actomyosin.

The supernatant was diluted to 0.03 M KCl during slow stirring and allowed to stand for 20 min. The myosin precipitate was collected by centrifugation at 1,400 x g for 20 min. The myosin precipitate was washed once with 0.03 M KCl and then dissolved in a minimum amount of 2 M KCl. The dissolved

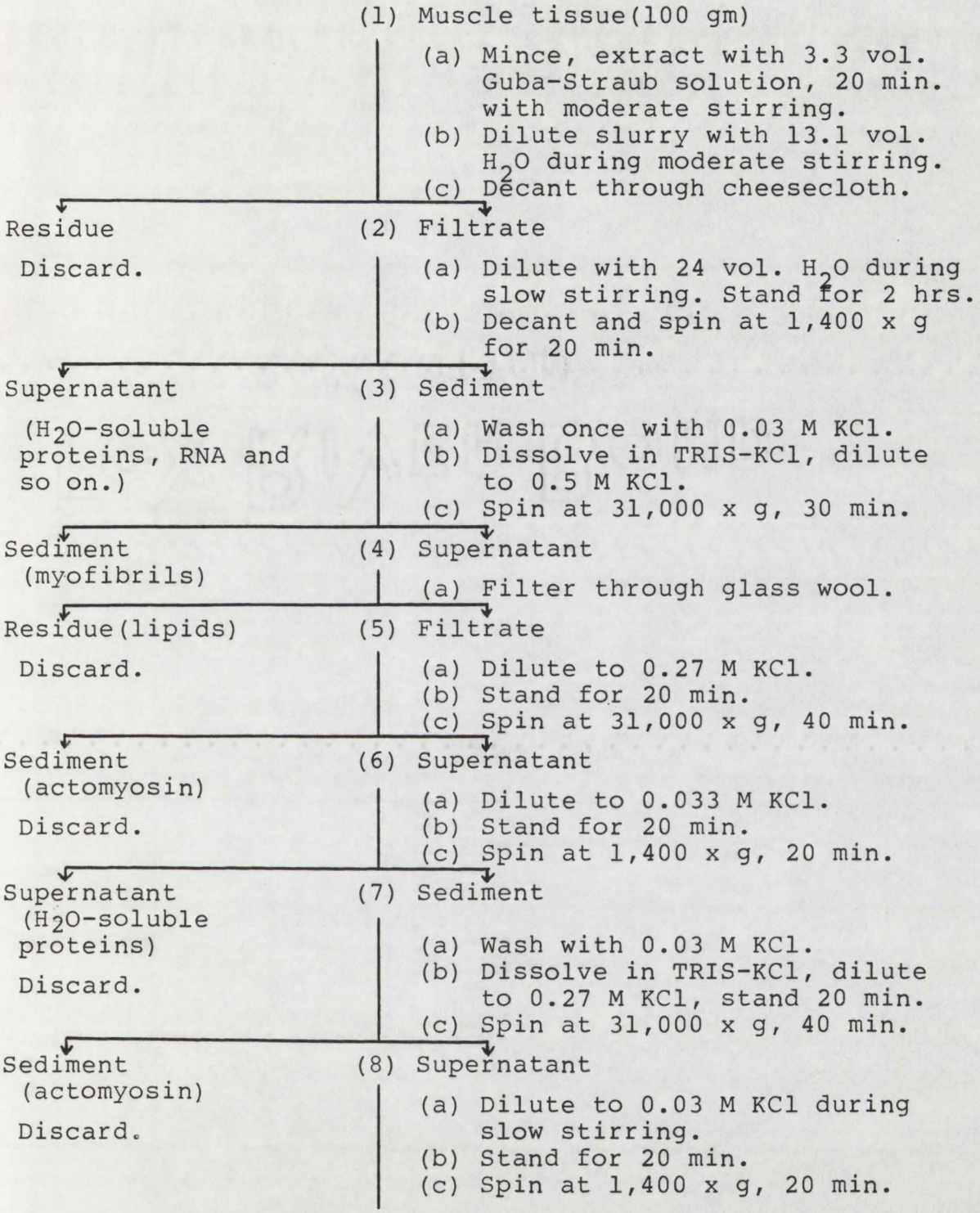


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Figure 6. Myosin preparation flow diagram.

Supernatant

Discard.

(9) Sediment

(a) Wash with 0.03 M KCl.
(b) Dissolve in 2 M KCl and dilute to 0.5 M KCl with 20 mM TRIS-acetate, pH 6.8.

(10) Purified myosin

(a) Mixed with an equal vol. of glycerol.
(b) Stored in -20 °C freezer.

Guba-Straub solution:

0.09M KH_2PO_4, 0.06 M K_2HPO_4, 0.3 M KCl, pH 6.5.

TRIS-KCl solution:

0.17 M TRIS-acetate, 1.3 M KCl, pH 6.8.

solution was then diluted to 0.5 M KCl by the addition of cold 20 mM Tris-acetate, pH 6.8. The purified myosin was mixed with an equal volume of cold glycerol and stored in a -20 °C freezer (Figure 6).

Protein Determination

The protein concentration was determined using the method of Lowry et al. (87). Lowry solution A (2% Na_2CO_3 in 0.1 N NaOH) was mixed in the ratio of 100 : 1 : 1 with Lowry solution B (1% $CuSO_4 \cdot 5H_2O$) and Lowry solution C (2% K-Na-tartrate) immediately before use to give Lowry solution D. Phenol solution was prepared immediately prior to use by diluting Folin and Ciocalteu's phenol reagent (Sigma Chemical Co., St. Louis, MO) 1 : 1 with water.

To assay for protein, 5 ml. of Lowry solution D was added to 1 ml. of appropriately diluted protein solution, and the mixture was incubated for 10 min. at room temperature. Then 0.5 ml. of the diluted phenol solution was added and mixed immediately. The mixture was allowed to stand for 45 min. at room temperature for color development. Absorbance was measured at 660 nm against a control consisting of distilled water plus all other reagents. The protein concentration was determined by comparison with a standard curve prepared from crystalline bovine serum albumin (Appendix figure 1).

Pretreatment of Hemoglobin Substrate

Crude hemoglobin powder (Sigma Chemical Co., St. Louis, MO) was dissolved in water and dialyzed against water for 48 hours. The hemoglobin solutions from all the cellophane tubes were mixed, and the precipitate which appeared during dialysis was removed by centrifugation. The supernatant solution was stored in a plastic container. It is possible to prepare enough dialyzed hemoglobin solution at one time for thousands of proteinase assays.

Cathepsin D Assay

Cathepsin D activity was assayed basically by the method of Anson (3) as modified by Whitaker and Seyer (157). The assay was initiated by the addition of 0.25 ml. aliquots of enzyme solution to 0.7 ml. of 50 mM sodium-acetate buffer, pH 3.8, containing 5 mg of hemoglobin substrate per ml. The reaction mixture was incubated at 37 °C. for 60 min. The reaction was then terminated by the addition of 0.7 ml. of 10% (w/v) trichloroacetic acid. Following centrifugation at 3,000 x g for 10 min. one milliliter of the supernatant was analyzed by the Folin-phenol method (87). One unit of enzyme activity was defined as a change in absorbance of 0.05 unit at 500 nm. after correction for any measured absorbance occurring in the absence of added enzyme.

Proteolytic Activity on Myofibrils

Assay procedure

Fifty microliters of cathepsin D (9 units/ml) was mixed with 200 µl. of incubation buffer (50 mM sodium-acetate buffer, pH 3.8, containing 0.02% sodium azide) and then preincubated for 5 min. at 37 °C. Bovine myofibrils (18 mg/ml) in a volume of 50 µl, were added to the mixture with an automatic pipette, and then incubated at 37 C for the appropriate time in a shaking water bath. At the end of the incubation period, 300 µl. of solubilization solution (50 mM Tris-glycine, pH 8.8, containing 1% (w/v) SDS, 1 mM dithiothreitol, 20% (v/v) glycerol, and 0.02% (w/v) pyronin Y) was added with agitation on a vortex mixer (93). The assay tubes were quickly covered with aluminum foil and immediately transferred to a boiling water bath for 10 min. Reagent blanks were prepared by substituting water for enzyme or by withholding the enzyme until just before heating (both methods yielded the same results in sodium dodecyl sulfate polyacrylamide gels).

Gel electrophoresis

Disc gel electrophoresis was performed according to the method of Davis (30). 1.2 ml. of the running gel solution (7.5% acrylamide) per tube, pH 8.9 (Tables 1 and 2) was added to the gel tube. Care was taken so that no air bubbles were trapped in the gels. After the tubes were filled with gel

solution, a water layer was placed on top of the gel solution in order to eliminate the meniscus and to obtain a flat smooth gel surface. The tubes were then placed directly under a day-light fluorescent light for about 30 min. Following photopolymerization, the water layer was removed and a stacking gel was prepared by adding about 0.15 ml. of stacking gel solution, pH 6.7 to the top of the running gel. A water layer was placed on top of the stacking gel as previously described.

Electrophoresis was conducted in a Bio-Rad Model 150 A gel electrophoresis cell with chamber buffer consisting of 0.44 M Tris-glycine. A Heathkit IP-17 constant voltage power supply of 0-40 voltage, 100 mA capacity was employed. 12-20 µg. of protein samples in the presence of 20% glycerol were run at a current of 3 mA per tube.

At the completion of electrophoresis (1-2 hours total run), the gels were removed from the tubes and the dye front was marked by piercing the gel with a hypodermic needle. The gels were then stained in a solution of 0.25% Coomassie Brilliant blue R-250 in methanol : acetic acid : water (5 : 1 : 5) for one hour or longer and were destained in a solution of 7.5% acetic acid and 5% methanol until the background was clear.

SDS electrophoresis was performed according to the method of Weber and Osborn (154) as modified by Porzio and Pearson (113). For the 10% acrylamide (100 : 1) gel system, a stock solution was prepared by dissolving 25.0 g. acryl-

amide and 0.25 g. bisacrylamide in water and bringing the final volume to 100 ml. Additional stock solutions were prepared as 2.0 M Tris-glycine (0.50 M Tris : 1.50 M glycine, pH 8.8), 50 % glycerol, 2.5% SDS/2.5 mM EDTA, 1% N,N,N',N'-tetramethylethylenediamine (TEMED), and 1% ammonium persulfate (Table 3). The casting solution consisted of stock solutions in the following proportions: 10 volumes acrylamide, 5 volumes Tris-glycine buffer, 1.25 volumes glycerol solution, 1 volume SDS/EDTA, 1 volume N,N,N',N'-tetramethylethylenediamine and 6.75 volumes water. One volume of the initiator, ammonium persulfate, was added immediately before casting. After the gels were loaded to within 1 cm. of the upper edge of the tube (5 x 90 mm), the surface of the acrylamide solution was layered with water to form a smooth interface. After polymerization was completed in approximately 30 min, the layering water was poured off and the gels were ready to use.

Electrophoresis was conducted in a Bio-Rad Model 150A gel electrophoresic cell with chamber buffer consisting of 200 mM Tris-glycine (pH 8.8) and 0.1% SDS in each chamber. Protein samples were loaded on the gel and entry of the sample into the gel was initiated at 0.5 mA per gel. After the sample had completely entered the gel, the current was raised to 1.0 mA per gel (12 mA at 40 volts, for 12 gels) and migration continued until the dye front was within 5 mm. of the bottom of the gel (5-6 hours total run). The gels were removed from the tubes, stained and destained as previously described.

Table 1. Stock Solutions for Disc Electrophoresis

(A)	
1 N HCl	24 ml
TRIS	18.3 gm
TEMED	0.12 ml
Water	to 100 ml
(pH 8.9)	
(B)	
1 N HCl	24 ml
TRIS	3.0 gm
TEMED	0.12 ml
Water	to 100 ml
(pH 6.7)	
(C)	
Acrylamide	30 gm
Bisacrylamide	0.8 gm
Water	to 100 ml
(D)	
Acrylamide	10 gm
Bisacrylamide	2.5 gm
Water	to 100 ml
(E)	
Ammonium persulfate	1.2 gm
Water	to 100 ml
(F)	
Riboflavin	6.0 mg
Water	to 100 ml
(G)	
Bromophenol blue	5.0 mg
Water	to 100 ml

Table 2. Working Solutions for Disc Electrophoresis

Running gel solution (pH 8.9)	Stacking gel solution (pH 6.7)	Stock buffer solution for reservoir (pH 8.3)	
3 parts A	3 parts B	TRIS	6.0 gm
3 parts C	3 parts D	Glycine	29.0 gm
1 part E	1 part F	Water	to 1 liter
5 parts water	5 parts G		

Table 3. Stock Solutions for SDS-Polyacrylamide Gel Electrophoresis

(A)	
TRIS-glycine solution	
0.5 M TRIS	60.5 gm
1.5 M Glycine	112.5 gm
Water	to 1 liter
(B)	
25% Acrylamide(0.25% Bisacrylamide)	
Acrylamide	62.5 gm
Bisacrylamide	0.625 gm
Water	to 250 ml
(C)	
2.5% SDS	
SDS	25.0 gm
Water	to 1 liter
(D)	
1% Ammonium persulfate	
Ammonium persulfate	0.1 gm
Water	to 10 ml
(E)	
Chamber buffer, pH 8.8	
2.0 M TRIS-Glycine	100 ml
2.5% SDS	40 ml
Water	to 1 liter
(F)	
Solubilization solution	
1% SDS	40 ml of (C)
0.005 M Na_2-EDTA	0.86 gm
0.05 M TRIS-Glycine	2.5 ml of (A)
0.01 M Dithiothreitol	0.0154 gm
20% Glycerol	20 ml
0.02% Pyronin Y	0.02 gm
Water	to 100 ml

Molecular weight estimates of hydrolysis products were made by comparing the mobilities of the polypeptide bands in SDS polyacrylamide gels with those of standard myofibrillar proteins (113).

Proteolytic Activity on Myosin

Assays were done by exactly the same manner as that for myofibrils except that beef myosin (2.44 mg/ml) in a volume of 100 µl was used.

Preparation of Pepstatin-Sepharose Column

Pepstatin was attached to Sepharose for affinity chromatography by a modification of the method of Kregar et (74). All initial steps were performed at 25 °C. Fifty milligrams of pepstatin were suspended in 3 ml. of methanol and mixed with 30 ml. of 0.025 N NaOH. Three grams of AH-Sepharose 4-B was washed with 600 ml. of 0.5 M NaCl to remove the added lactose and dextran followed by rinsing with water to remove NaCl. The water-rinsed AH-Sepharose 4B was then added to the pepstatin suspension after which 500 mg. of 1-ethyl-3(3-dimethylaminopropyl) carbodiimide were added. The pH was adjusted and maintained at 6.2. After 80 min., 500 mg. more of 1-ethyl-3(3-dimethylaminopropyl) carbodiimide were added and the pH was maintained at 6.2 for another 40 min. The solution was then held for 24 hours at 4 °C. and then washed

successively with 100 ml. quantities of 0.1 M sodium-acetate buffer (pH 5.0), 0.1 M Tris-HCl buffer (pH 8.6), 0.05 M sodium-acetate buffer (pH 3.5), and 0.1 M sodium-acetate buffer (pH 5.0), each of which contained 1.0 M NaCl. The pepstatin-Sepharose solution was then ready for use or could be stored in the pH 5.0 buffer at 4 °C.

Cathepsin D Purification Procedure

Cathepsin D was extracted from bovine skeletal muscle by a modification of the method of Smith and Turk (132). Muscle tissue, taken within 1 hour of slaughter, was transported to the laboratory on ice. All subsequent operations were carried out at 4 °C.

Preparation of crude extract

Seventy-five grams of muscle tissue, freed of blood vessels and fat, were homogenized with 4 volumes of cold water. Cellular debris was removed from the homogenate by centrifugation at 19,000 x g for 60 min. in a JA-14 rotor (Beckman Model J-21C regrigenated centrifuge). The supernatant was filtered through 8 layers of cheese cloth to remove the residual fat particles.

Ammonium sulfate fractionation

The protein in the homogenate supernatant was precipitated by the addition of solid ammonium sulfate until the solution was 40% saturated with the ammonium sulfate. After 30 min., the precipitate was removed by centrifugation at 13,200 x g for 30 min. and discarded. The supernatant was then brought to 70% saturation with ammonium sulfate, and allowed to stand for 30 min. The precipitate was collected by centrifugation at 13,200 x g for 30 min. The supernatant was discarded.

Chromatography on pepstatin-Sepharose column

The 40-70% saturated ammonium sulfate fraction was resuspended in a small amount of water and the resuspended precipitate was dialyzed overnight against water. The precipitate which appeared during dialysis was removed by centrifugation and discarded. The supernatant was adjusted to pH 4.0 (0.1 M sodium-acetate, 1.0 M NaCl) by the addition of concentrated buffer with stirring and the small amount of precipitate which formed was centrifuged off.

The supernatant solution was then applied to a column (0.7 x 18 cm) of pepstatin-Sepharose which had been pre-equilibrated with the same buffer. After washing with 80 ml. of 0.1 M sodium-acetate buffer (pH 5.0) containing 1.0 M NaCl, The enzyme was eluted with 0.1 M Tris-HCl buffer (pH 8.6)

containing 1.0 M NaCl. Fractions of 3 ml. were collected and assayed for activity.

RESULTS

Myofibril Preparation

A typical result of SDS-polyacrylamide gel electrophoresis of the isolated bovine myofibrils is shown in Figure 7, and was nearly identical to that of SDS-gels of rabbit myofibrils (113). Inspection of Figure 7 showed good resolution as illustrated by the separation of M-lines α and β (41). The myosin heavy chain entered and migrated as a narrow symmetrical band. The separation of myosin heavy chain from M-lines α and β with whole myofibril samples can be used as an internal check of the efficiency and consistency of the particular set of gels prepared for routine separations.

Purification of Cathepsin D

Table 4 summarizes a typical purification procedure for cathepsin D from 75 grams of bovine skeletal muscle tissue. The final preparation had a specific activity which was routinely between 34 and 42 units/mg of protein and showed a single protein band in polyacrylamide gel electrophoresis in the presence of 0.1% sodium dodecyl sulfate (Figure 11).

Proteolytic Activity of Cathepsin D on Myofibril Proteins

Samples of fresh myofibrils that were incubated with

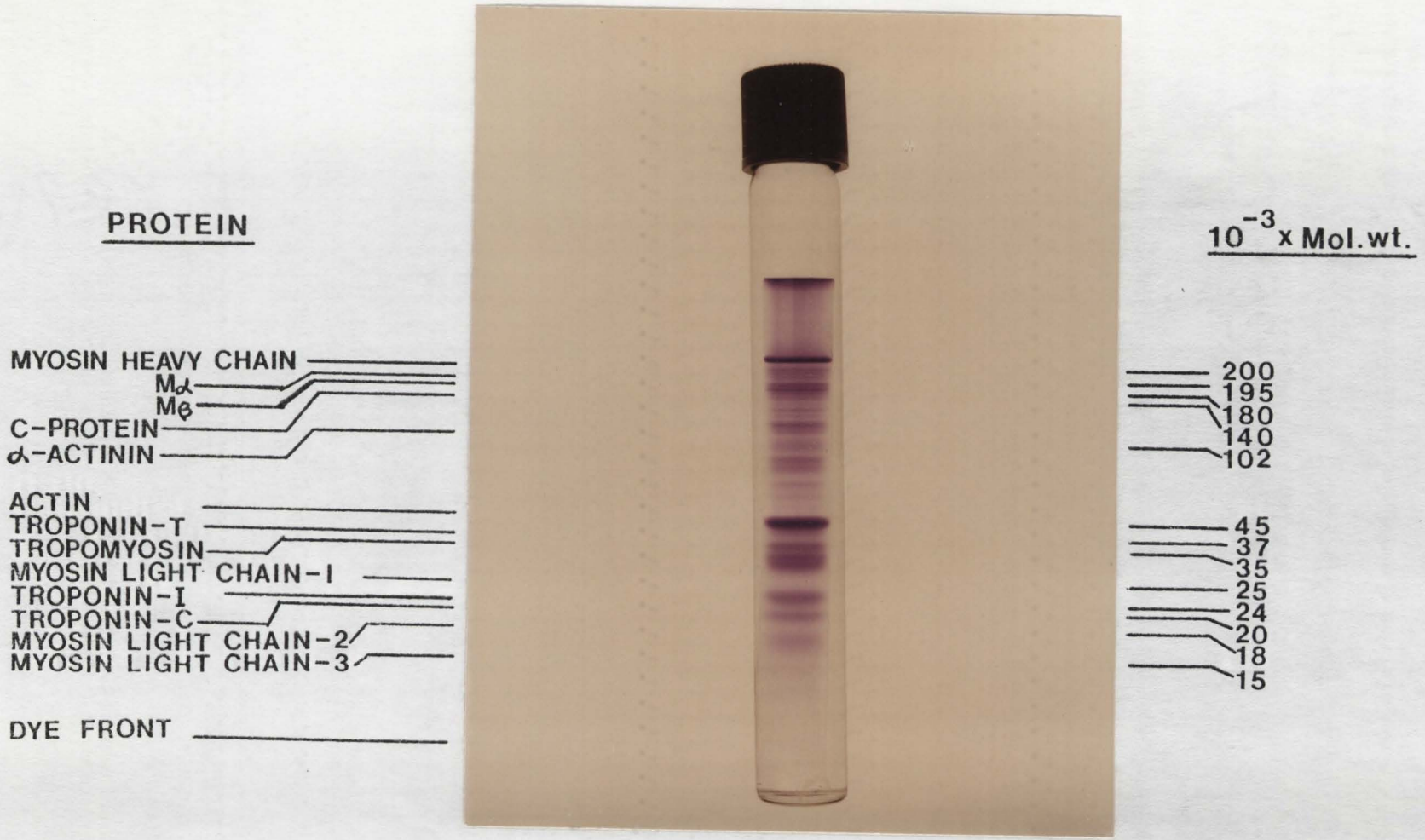
cathepsin D at intervals from 0 hour to 4 hours showed progressive changes in their protein patterns (Figure 13). There was an increase of components having molecular weights of about 170,000, 150,000, 120,000, 80,000 and 70,000 daltons, and the 200,000 dalton myosin heavy chain appeared to be degraded. The other major proteins of myofibrils, including M-lines α and β, C-protein, actin as well as myosin light chains were also degraded as shown by the appearance of the lower molecular weight components as the enzyme-substrate incubation time increased. Actin was less readily degraded than myosin.

Proteolytic Activity of Cathepsin D on Myosin

A typical result of SDS-polyacrylamide gel electrophoresis of the isolated myosin is shown in Figure 8. When this isolated myosin was incubated with bovine muscle cathepsin D at intervals from 0 hour to 4 hours, progressive changes in the myosin heavy chain protein band were observed (Figure 14). Inspection of Figure 14 showed that there were four major proteolytic products of 110,000, 107,000, 80,000 and 70,000 daltons molecular weight respectively,in the 30 min. gel (gel H). After one hour of incubation, the 110,000 and 107,000 daltons protein bands (gel I) were less intense than in the sample incubated for 30 min. (gel H). The 80,000 dalton protein band was similar to the 30 min. gel (gel H) and the 70,000 dalton protein band became more intense.

Only a faint 120,000 dalton proteolytic band was apparent in this gel. For the 2 hour gel (gel J), all four major proteolytic bands (110,000, 105,000, 80,000 and 70,000 daltons molecular weight) became less intense, indicating further proteolysis was occurring. However, the 120,000 dalton band became more intense. Furthermore, a new proteolytic product of 60,000 dalton became more intense in the gel after 2 hours incubation. The myosin heavy chain band became progressively less intense as the substrate-enzyme incubation time increased. For the 4 hour gel (gel K), the myosin heavy chain band had almost completely disappeared and three major proteolytic bands of 120,000, 70,000 and 40,000 daltons respectively, were observed. Gels G and L were the 0 hour and 4 hour control (without enzyme), respectively. No changes in the protein band patterns were observed in either of these two controls.

Figure 7. Polyacrylamide gel electrophoresis of isolated beef myofibrils in sodium dodecyl sulfate. A myofibril sample of approximately 75 µg in 50 µl was used per gel. The method is described under "EXPERIMENTAL PROCEDURES".


PROTEIN
MYOSIN HEAVY CHAIN
Mα
Mβ
C-PROTEIN
α-ACTININ
ACTIN
TROPONIN-T
TROPOMYOSIN
MYOSIN LIGHT CHAIN-1
TROPONIN-I
TROPONIN-C
MYOSIN LIGHT CHAIN-2
MYOSIN LIGHT CHAIN-3
DYE FRONT
10^-3 x Mol.wt.
200
195
180
140
102
45
37
35
25
24
20
18
15

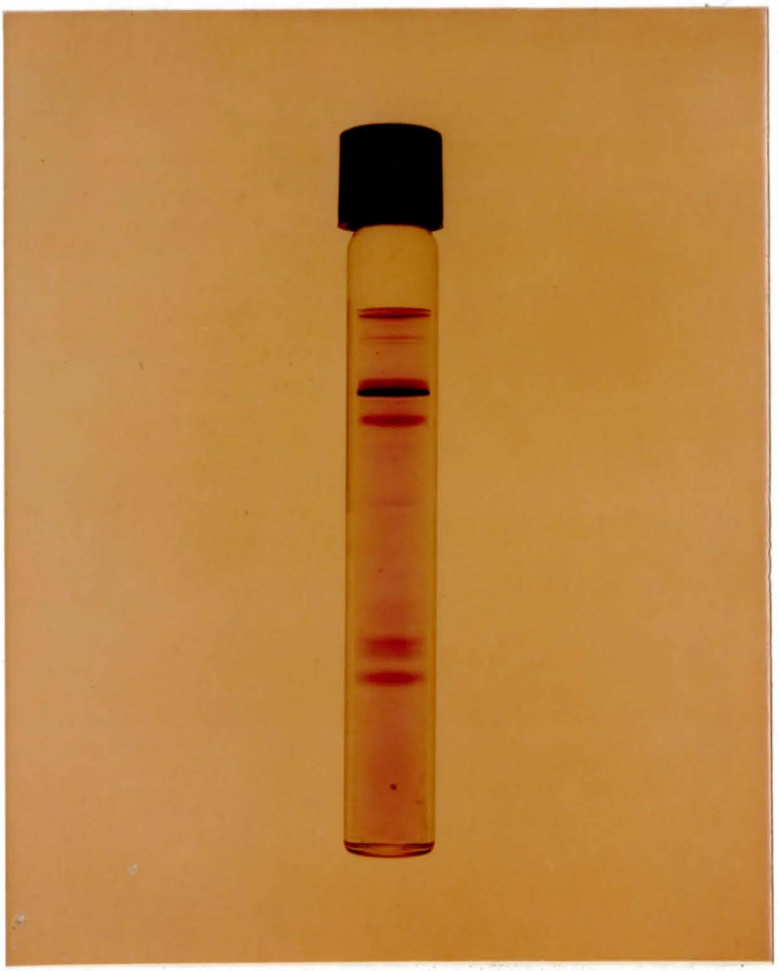


Figure 8. Polyacrylamide gel electrophoresis of isolated beef myosin in sodium dodecyl sulfate. A myosin sample of approximately 8 µg in 10 µl was used per gel. The method is described under "EXPERIMENTAL PROCEDURES".

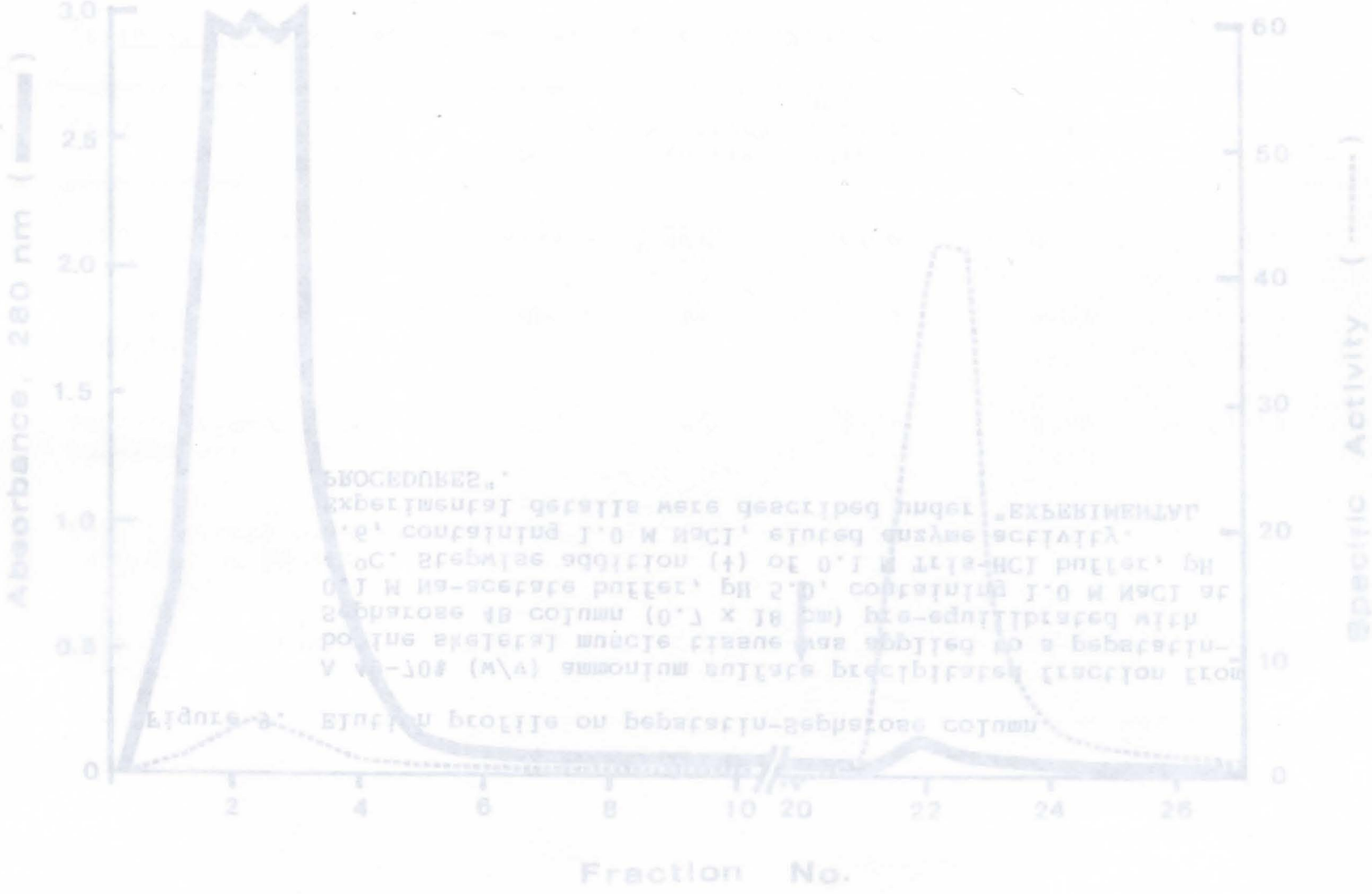

Absorbance, 280 nm (——)
Specific Activity (------)
Fraction No.
0
0.5
1.0
1.5
2.0
2.5
3.0
10
20
30
40
50
60
2
4
6
8
10
20
22
24
26

Figure 9. Elution profile on pepstatin-Sepharose column.

A 40-70% (w/v) ammonium sulfate precipitated fraction from bovine skeletal muscle tissue was applied to a pepstatin-Sepharose 4B column (0.7 x 18 cm) pre-equilibrated with 0.1 M Na-acetate buffer, pH 5.0, containing 1.0 M NaCl at 4 ^{o}C. Stepwise addition (↓) of 0.1 M Tris-HCl buffer, pH 8.6, containing 1.0 M NaCl, eluted enzyme activity. Experimental details were described under "EXPERIMENTAL PROCEDURES".

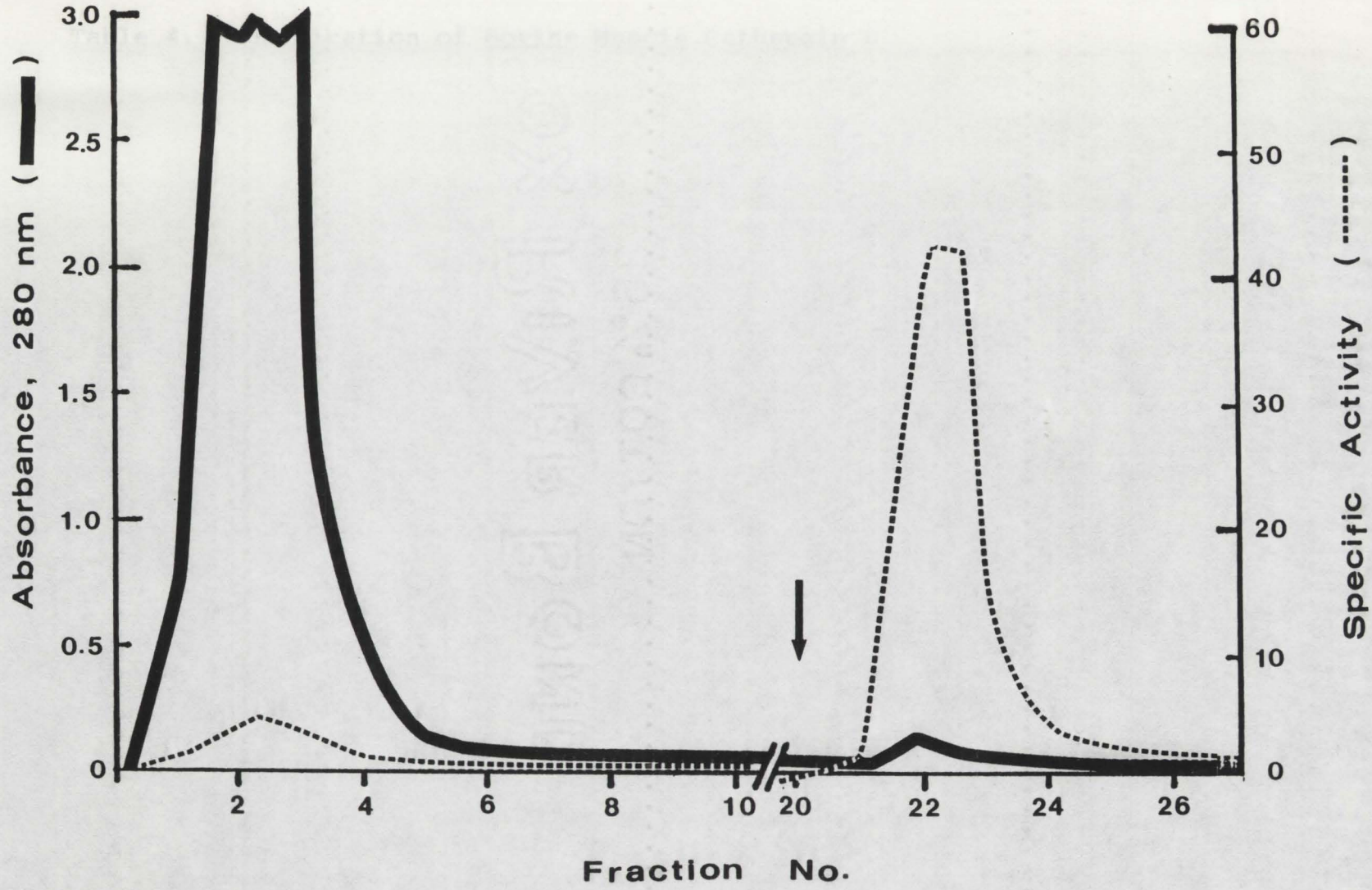

Absorbance, 280 nm (——)
3.0
2.5
2.0
1.5
1.0
0.5
0
60
50
40
30
20
10
0
Specific Activity (--------)
2
4
6
8
10
20
22
24
26
Fraction No.

Table 4. Purification of Bovine Muscle Cathepsin D

Step	Total Protein (mg)	Total Activity (units)	Specific Activity (units/mg)	Purification	% Recovery
Crude Extract	4732	3150	0.66	____	100
40-70% Saturated Ammonium Sulfate	980	230	0.23	0.35	7.3
Post-Dialysis Supernatant	633	450	0.71	1.08	14
Post-Dialysis Supernatant Adjusted to pH 4.0	54	86	1.59	2.41	2.73
Pepstatin-Sepharose Eluate	1.64	62	38	57.3	1.97

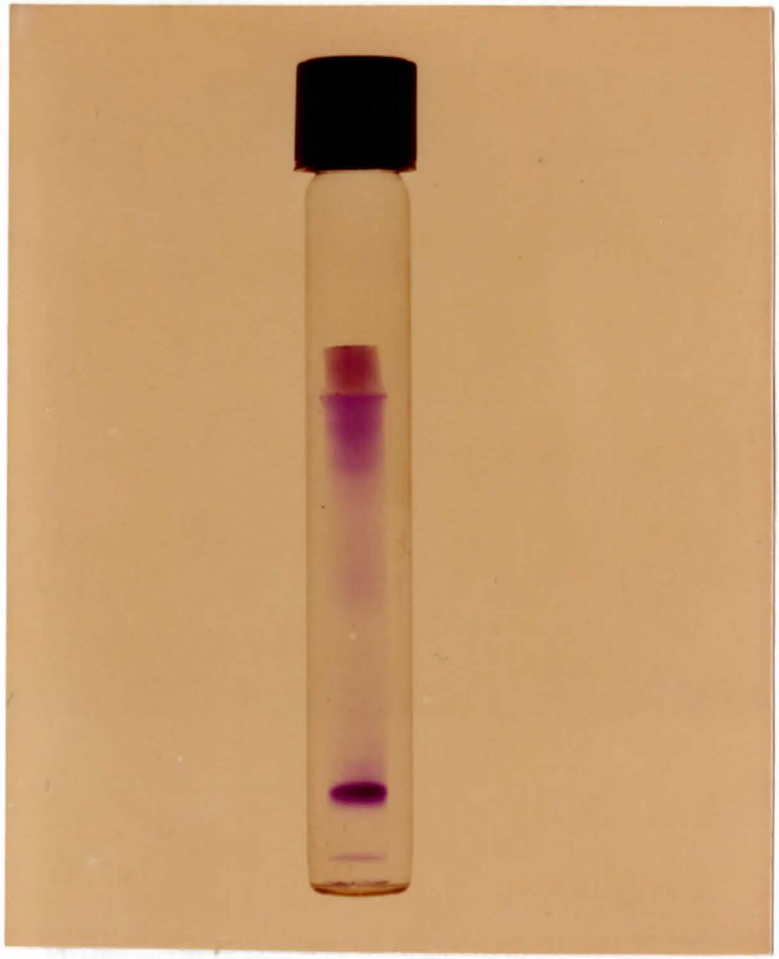


Figure 10: Discontinuous polyacrylamide gel electrophoresis of isolated bovine muscle cathepsin D. 15 ug of the final preparation in 0.1 M Tris-HCl buffer (pH 8.60) was used per gel. The method is described under "EXPERIMENTAL PROCEDURES".

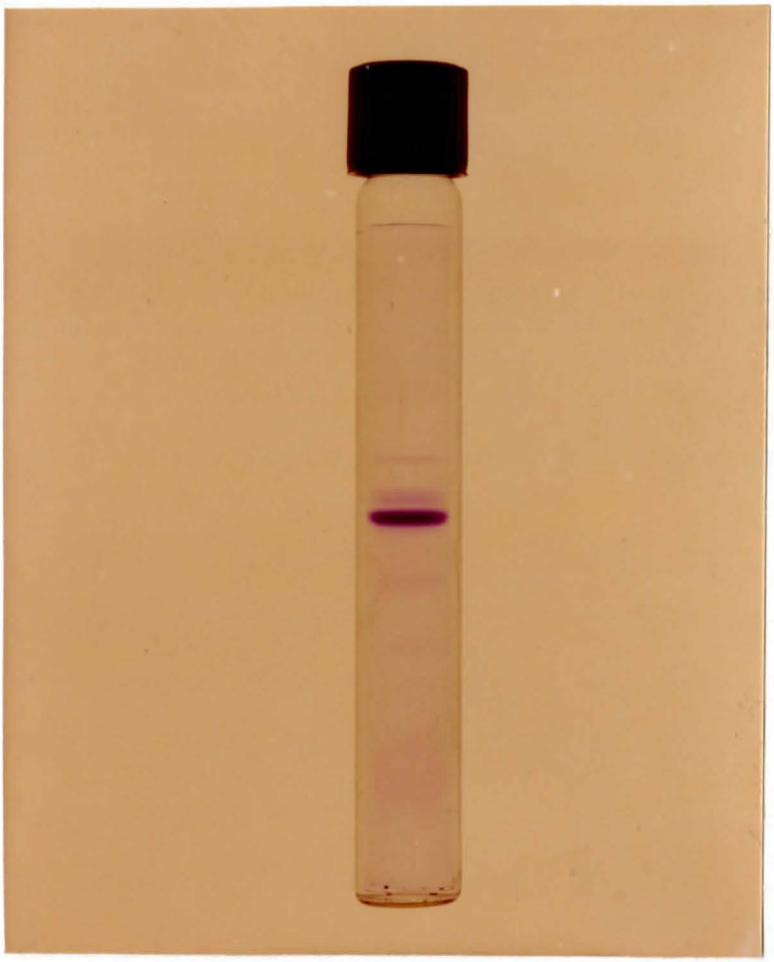


Figure 11. Polyacrylamide gel electrophoresis of isolated bovine muscle cathepsin D in sodium dodecyl sulfate. 12 µg of the final preparation was used per gel. The method is described under "EXPERIMENTAL PROCEDURES".

Figure 12. Estimation of bovine muscle cathepsin D subunit molecular weight by electrophoresis on polyacrylamide gel in sodium dodecyl sulfate. 12 µg of the final preparation was applied to the gel. The method is described under the "EXPERIMENTAL PROCEDURES". The arrow indicates the position of cathepsin D.

Abbreviations:	Mol. wt
PHB = phosphorylase b	94,000
BSA = bovine serum albumin	67,000
EWO = egg white ovalbumin	43,000
CA = carbonic anhydrase	30,000
TI = trypsin inhibitor	20,100

—Figure 12 continued on next page—

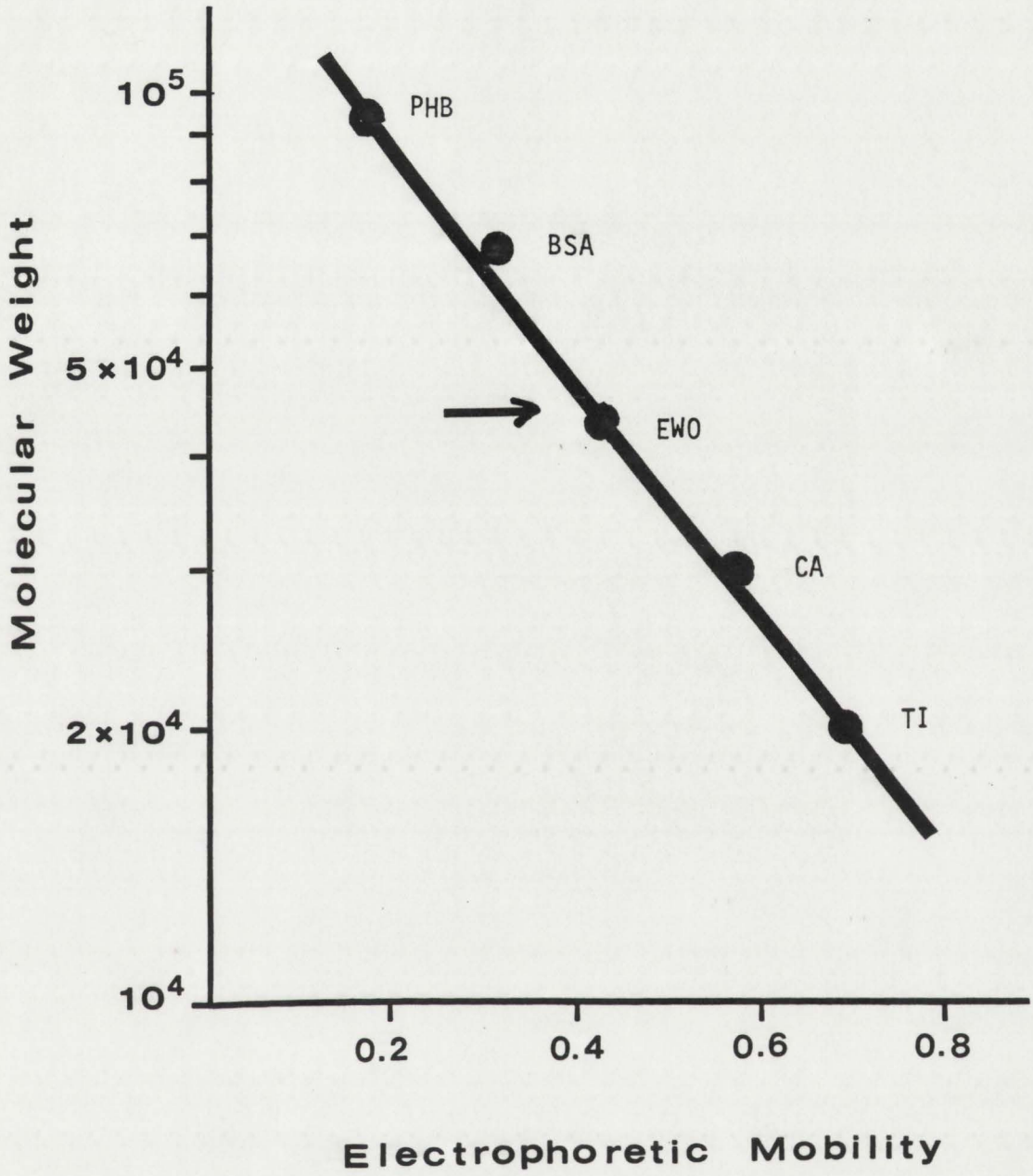

Molecular Weight
10^5
5x10^4
2x10^4
10^4
PHB
BSA
EWO
CA
TI
0.2
0.4
0.6
0.8
Electrophoretic Mobility

Figure 13. Degradation of beef myofibrillar proteins by bovine muscle cathepsin D.

Gel B: Myofibrils(18mg/ml) were incubated with cathepsin D(9 units/ml) at 37 °C in 50 mM sodium acetate buffer, pH 3.8, containing 0.02% sodium azide. Assay was terminated at 30 min. after reaction by treatment with solubilization solution as described under "EXPERIMENTAL PROCEDURES". Samples(50 µl), equivalent to 75 µg of substrate protein, were subsequently applied to the 10% gel and electrophoresis in the presence of 0.1% SDS was performed.

Gels C,D and E: Reaction times were 1, 2, and 4 hours, respectively. All other conditions were as described for gel B.

Gels A and F: Reaction times were 0 and 4 hour control, respectively. All other conditions were as described for gel B except for substituting water for enzyme.

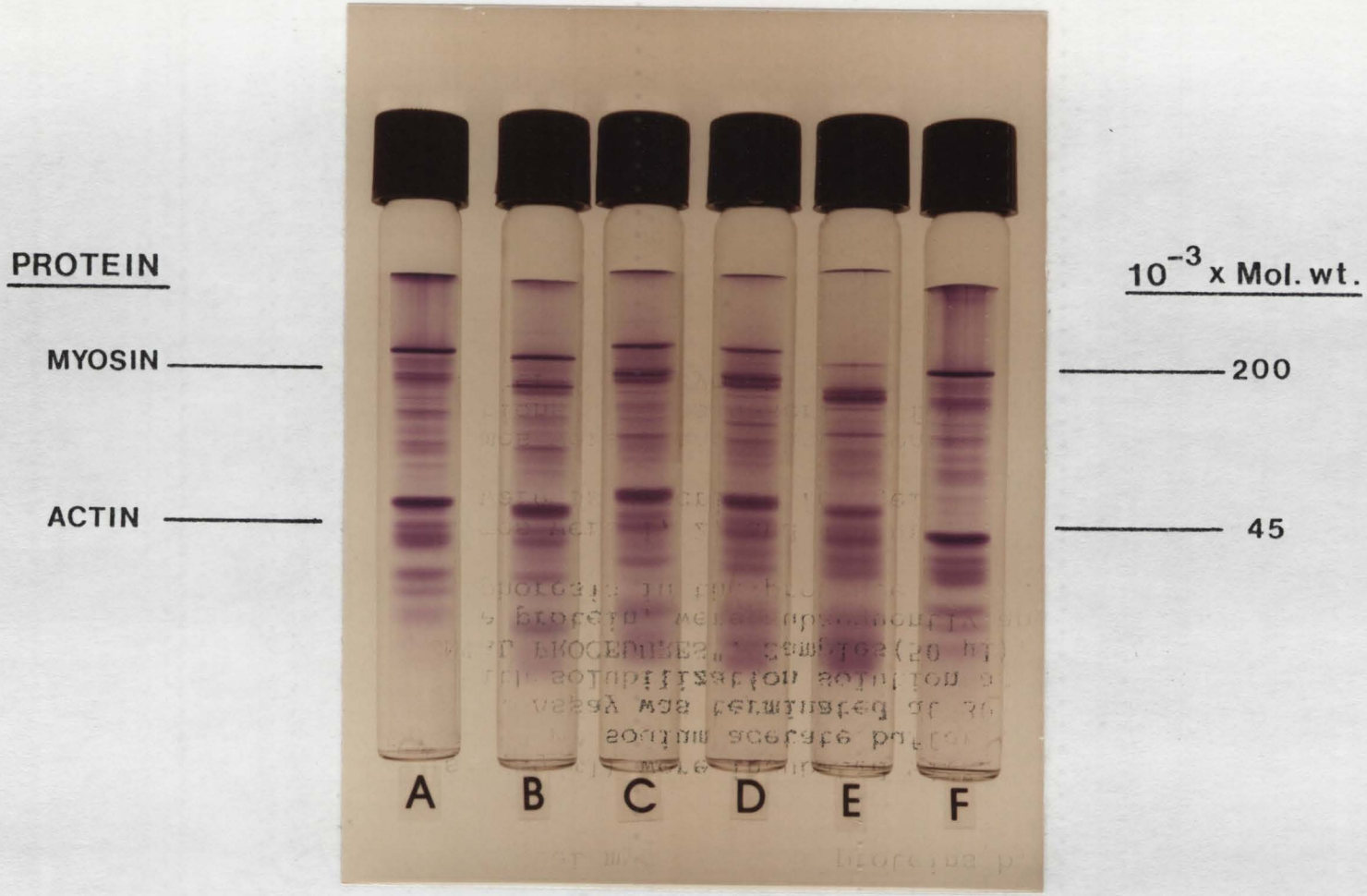

PROTEIN
MYOSIN
ACTIN
10^{-3} x Mol. wt.
200
45
A
B
C
D
E
F

Figure 14. Degradation of beef myosin by bovine muscle cathepsin D.

Gel H: Myosin(2.44mg/ml) was incubated with cathepsin D(9 units/ml) at 37 °C in 50 mM sodium acetate buffer, pH 3.8, containing 0.02% sodium azide. Assay was terminated at 30 min. after reaction by treatment with solubilization solution as described under "EXPERIMENTAL PROCEDURES". Samples(25 µl), equivalent to 9 µg of substrate protein, were subsequently applied to the 10% gel and electrophoresis in the presence of 0.1% SDS was performed.

Gels I,J and K: Reaction times were 1, 2 and 4 hours, respectively. All other conditions were as described for gel H.

Gels G and L: Reaction times were 0 and 4 hour control, respectively. All other conditions were as described for gel H except for substituting water for enzyme.


MYOSIN
10^{-3} x Mol. wt.
200
120
110
107
80
70
60
45
G
H
I
J
K
L

DISCUSSION

Purification of Cathepsin D

The precipitation steps with ammonium sulfate were used primarily for concentration of the enzyme fractions to a volume suitable for application to the small pepstatin-Sepharose column. The enzyme losses in these stages were appreciable and often little purification resulted (Table 4). If some preliminary purification with high yield is applicable, then another method may be more satisfactory in increasing the yield of cathepsin D. The increase in percent recovery of cathepsin D in the post-dialysis supernatant (Table 4), compared to the previous step, may be due to the release of enzyme from either membrane sites or from unidentified inhibitors (52, 132).

Affinity chromatography on immobilized pepstatin was very effective in removing impurities, as reflected by the increase of specific activity and the sharp decrease of total proteins. Thus, it affords a rapid and simple purification of cathepsin D. A typical elution diagram for bovine muscle cathepsin D is shown in Figure 9. Cathepsin D was bound to immobilized pepstatin at pH 5.0 in 0.1 M sodium-acetate buffer and 1.0 M NaCl. Under these conditions, nearly all of the impure proteins passed through the column (Figure 9). Change of buffers to pH 8.6 in 0.1 M Tris-HCl containing 1.0 M NaCl affected a prompt release of enzyme of strong proteolytic activity.

It was reported that the presence of a high salt concentration during affinity chromatography is essential (132). In experiments in 0.1 M sodium-acetate buffer without added

NaCl, none of the proteolytic activity was retained on the resin at pH 5.0. However, in the presence of 1.0 M NaCl almost all of the inactive proteins pass through the column at pH 5.0 while the bulk of the enzyme is bound and elutes when the pH is brought to 8.6.

Purity of Cathepsin D

Polyacrylamide disc gel electrophoresis gave useful information about the purity of the preparation because at the pH of these gels, cathepsin D yields a single band (Figure 10). Polyacrylamide gel electrophoresis in 0.1% SDS yields one major protein band (Figure 11). This result in both the SDS- and disc-polyacrylamide gel electrophoresis gave strong evidence that this enzyme has not been subjected to concomitant autolysis or proteolysis by other proteases during purification.

Subunit Molecular Weight of Cathepsin D

Bovine muscle cathepsin D shows a single protein band after polyacrylamide gel electrophoresis in sodium dodecyl sulfate (Figure11). A single protein band suggests that this enzyme has not been subjected to protease action during purification. The subunit molecular weight estimated from six protein standards is 42,000 ± 1,000 (Figure 12). This value is higher than that found for bovine uterus enzymes (123) and lower than that found for bovine spleen enzyme (114), but agrees well with that of the bovine brain enzyme (132, 157), rat liver and skeletal muscle enzymes (126). This subunit molecular weight suggests that the native enzyme is a monomer.

Proteolytic Activity of Cathepsin D on Myofibril Proteins

Inspection of Figure 13 showed that there was very little difference between the 30 min. (gel B) and 60 min. (gel C) enzyme-treated myofibrils, but a sharp difference was observed in the gel patterns between 30 min. (gel B) and 4 hour (gel E) enzyme-treated myofibrils. This demonstrated that as the incubation time increased, the myofibrillar proteins were degraded more extensively by cathepsin D.

The finding that myofibrillar proteins were degraded by cathepsin D was supported by several reports in which essentially the same results were found (6, 48, 118, 126, 135, 139, 140). Schwartz and Bird (126) reported that muscle and liver cathepsin D from rat degraded the heavy chains of isolated myosin (200,000 daltons) to fragments of about 175,000 daltons to 150,000 daltons and then subsequently to fragments of 100,000 daltons. The same authors also reported that cathepsin D degraded isolated F-actin at about 10% the rate of attack on myosin heavy chain. Arakawa et al. (6) reported that cathepsin D degraded the heavy chains of myosin present in myofibrils to fragments of 150,000 daltons and 70,000 daltons. Suzuki et al. (139, 140) reported that purified cathepsin D from rabbit longissimus dorsi muscle hydrolyzed myosin, actin and actomyosin, as well as water-soluble muscle proteins. Robbins et al. (118) showed that cathepsin D from muscle and spleen degraded myofibrils under postmortem pH

conditions (5.1-5.3) causing an alteration of Z disk structure and breakdown of myosin heavy and light chains as well as effecting changes in the troponin-tropomyosin complex. Some degradation of α-actinin was evident at 37 °C.

The origin of the changes leading to the alteration of Z disk structure when myofibrils were treated with cathepsin D was uncertain, and it was difficult to rationalize this breakdown with the degradation in myofibrillar proteins observed in this study. However, some recent observation on the breakdown of myofibrillar proteins by lysosomal proteases might provide a possible explanation for the observed changes in the Z disk structure. Schwartz and Bird (126) reported that cathepsin D degraded isolated F-actin at about 10% the rate of attack on myosin, and Spanier et al. (135) have demonstrated that myosin and actin could also be degraded in intact myofibrils or myofilaments by lysosomal proteases from normal muscle at pH 5.0. The effect was greater with treatment by lysosomal extracts from dystrophic muscle in which catheptic activity and acidic autolysis were known to increase (155). Robbin et al. (118) also demonstrated that lysosomal proteases from spleen degraded actin in myofibrils, and in isolated actomyosin complex (Walker, J., unpublished observations). Several studies (137, 141) suggested that the breakdown of actin with or without a concomitant breakdown of α-actinin could explain the loss of Z disk structure in myofibrils since it was possible that α-actinin binds to the F-actin thin filaments of adjacent sarcomeres in this region (118).

The studies with CAF demonstrated that this endogenous muscle proteinase could degrade certain of the myofibrillar proteins, but not myosin and actin (126). The present study, as well as those of Schwartz and Bird (126), Arakawa et al. (6), and Spanier et al. (135) demonstrates that another endogenous muscle proteinase, cathepsin D, is also capable of acting on myofibrillar proteins and has wider proteolytic activity than CAF.

It should be mentioned here that several publications (32, 104, 107) have shown that CAF was able to remove Z disks from intact myofibrils, degrade troponin, tropomyosin and C-protein components of muscle. It is likely that intralysosomal digestion under physiological conditions would require initial disruption of the myofibrils by CAF (32, 104), mechanical forces (49, 97) including electrical stimulation (22, 99, 125, 134, 160), and/or other factors. The myofilaments could then be sequestered and digested within sarcoplasmic reticulum components, which appears to be part of the lysosomal apparatus in skeletal muscle (17). In pathological states such as dystrophy (155) and ischemia (62), the stimulation of the lysosomal system and/or membrane labilization and enzyme release which may occur could result in extensive damaged by the lysosomal proteinases.

Up to the present time, the relative contribution of these two proteinases (cathepsin D and CAF) to the postmortem conditioning process of meat is unclear. CAF with its maximum activity at physiological pH (7.5) would not be expected to

play a significant role in myofibrillar protein breakdown under postmortem pH conditions, whereas cathepsin D which is active under postmortem pH conditions would be expected to play a major role (38, 134).

Proteolytic Activity of Cathepsin D on Myosin

As mentioned in the result section, the myosin heavy chain band became progressively less intense as the substrate-enzyme incubation time increased. For the 4 hour gel (gel K), the myosin heavy chain band had almost completely disappeared and three major proteolytic bands of 12,000, 70,000 and 40,000 daltons respectively, were observed (Figure 14).

Inspection of Figure 14 showed that degradation in vitro of the myosin heavy chain was clearly demonstrated with SDS-polyacrylamide gel electrophoresis. In addition to the myosin heavy chain band, other protein bands such as M-lines α and β, C-protein and protein bands with molecular weights greater than 200,000 daltons were also degraded as indicated by the decreased intensity of these bands, the increased intensity of the 120,000 daltons proteolytic band, and the appearance of several very faint low molecular weight proteolytic bands with increasing incubation time.

Many other problems remain to be solved before the underlying proteolytic mechanisms between myofibrils and cathepsin D can be fully understood. Apart from knowledge of the proteolytic activity of cathepsin D, where the kinetics involved in substrate-enzyme require further study, more work has to be done on the molecular level as well as on the microscopic level. The nature and specificity of cathepsin D has to be investigated on the molecular level. On the microscopic level it is essential to relate the network structures of myosin and other myofibrillar proteins with properties like three dimensional conformation and substrate-enzyme interaction. Without such knowledge it will not be possible to gain full insight into what happens during structure formation and a knowledge necessary for controlling and optimizing enzyme activity.

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APPENDIX

Figure 1. Standard curve for the determination of protein by the method of Lowry et al. (87)