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ABSTRACT

A COMPARISON OF TWO ENDOGENOUS SKELETAL MUSCLE PROTEASES AND AN EXOGENOUS PROTEASE OF BACTERIAL ORIGIN, PSEUDOMONAS PEROLENS: EFFECTS AND ACTIVITIES

By

George Joseph Seperich

These studies were undertaken to compare the activity of a Pseudomonas perolens protease with the activity of an endogenous proteolytic enzyme in muscle, the calcium activated sarcoplasmic factor (CASF). During the course of these studies the presence in muscle tissue of another proteolytic enzyme, the kinase activating factor (KAF), necessitated a comparison of these two sarcoplasmic enzymes and the bacterial protease. The activity of these enzymes on various substrates and on myofibrillar integrity and the possibility of establishing a hypothetical role in protein turnover or tissue protein degradation by the sarcoplasmic proteinases was assessed.

A protease released by Pseudomonas perolens ATCC 10757 was produced through a dense innoculum of the casamino acid medium supplemented with 10^{-5}M $ZnCl_2$ and 4.5 x 10^{-3}M $CaCl_2$. After growth at 10°C for 60 hours the protease was isolated by DEAE-Sephadex A-50 batch adsorption, ammonium sulfate precipitation and Sephadex G-100

molecular exclusion chromatography. This procedure yielded 0.01% protein recovery but a 967 fold purification of the enzyme.

The enzyme was more sensitive to the presence of EDTA than previously reported (50% inhibition occurred between 5 and 10 μmoles EDTA). Addition of $CaCl_2$ did not completely reverse the EDTA inhibition. The kinetic parameters of the enzyme, K_m and V_{max} (determined on N-CBZ-glycyl-L-leucine) were 2.6 mM and 169.4 μM leucine $ml^{-1}min^{-1}$, respectively. A molecular weight between 35,000-40,000 daltons was determined by SDS gel electrophoresis.

The activity of the above bacterial protease was compared to proteases isolated from rabbit skeletal muscle. The proteases were the calcium activated sarcoplasmic factor (Busch et al., 1972) and the kinase activating factor (Huston and Krebs, 1968).

The kinase activating factor (KAF) demonstrated proteolytic activity as well as the ability to activate phosphorylase kinase. SDS gel electrophoresis disclosed two components with molecular weights of 95,000-100,000 and 30,000-35,000 daltons.

The calcium activated sarcoplasmic factor (CASF) was isolated from rabbit skeletal muscle affected by two different treatments. Since this protease was implicated in muscle protein turnover it was decided to ascertain whether the amount of enzyme and/or its activity was increased under conditions of fasting. The effects of fasting were monitored by live animal weight, serum glucose level, serum nonesterified free fatty acid level and total free serum amino acid level. Additionally, the muscle weights for the semitendinosus and longissimus muscles and any attendant changes in

total, myofibrillar, sarcoplasmic and stromal protein and non-protein nitrogen components were assayed.

Serum glucose and total free amino acid levels remained approximately the same for the fed and fasted rabbits. Non-esterified free fatty acids were higher for the fasted than for the ad libitum fed rabbits. Total protein and the individual protein components of skeletal muscle were all much higher in the controls than in the fasted rabbits.

A greater amount of enzyme, CASF, was obtained from the fasted rabbit than from the control rabbit muscle, but the specific activity of the enzyme was similar although slightly higher for the fasted group. Catheptic activity was detected in each fraction, also. SDS gel electrophoresis of the enzyme from both treatments demonstrated subunits at molecular weights similar to those listed for the KAF.

A comparison of the relative enzymic activities demonstrated by these proteases yielded the following results.

1. The Pseudomonas perolens protease had the highest proteolytic activity of the compared proteases followed in decreasing order of activity by CASF (Fasted), CASF (Fed) and KAF.

2. The phosphorylase kinase activating ability was the highest for CASF (Fasted) followed by CASF (Fed) and KAF which were similar. Pseudomonas perolens protease had the lowest activity.

3. The Pseudomonas perolens protease was the only protease that hydrolyzed the substrate N-CBZ-glycyl-L-leucine.

4. KAF was the only protease completely inhibited by bovine heart inhibitor of kinase activating factor. CASF (Fasted) was the second most affected protease

followed by CASF (Fed). Pseudomonas perolens protease was unaffected by the inhibitor.

5. The protease of Pseudomonas perolens exhibited the most disruptive influence upon the integrity of myofibrils as observed by phase contrast microscopy and SDS gel electrophoresis. CASF (Fasted), CASF (Fed) and KAF were identical in influence. These proteases displayed an ability to disrupt and/or remove the Z-disc from the myofibril.

It was found that the fasting state did not increase CASF production of activity of the isolated enzyme significantly. The physiological and biochemical changes that occurred in the fasting study could not be accounted for by either CASF or KAF enzymes. The changes that occurred were rather extensive yet neither enzyme displayed the ability to initiate changes beyond degradation of α-actinin and tropomyosin. The bacterial protease caused extensive myofibrillar degradation, but its presence in vivo is difficult to rationalize.

A comparison of KAF and CASF activity on myofibrillar tissue, as observed microscopically and electrophoretically, in conjunction with the KAF inhibitor study demonstrated a similarity between these two enzymes. The SDS gel electrophoresis of KAF and CASF and their activity on casein and phosphorylase kinase demonstrated this similarity also, especially when these two enzymes were compared to any of the Pseudomonas perolens protease results.

A COMPARISON OF TWO ENDOGENOUS SKELETAL MUSCLE PROTEASES AND AN EXOGENOUS PROTEASE OF BACTERIAL ORIGIN, PSEUDOMONAS PEROLENS: EFFECTS AND ACTIVITIES

By

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I. INTRODUCTION

Proteolysis as an area of research has stimulated much interest for a number of reasons. The intrusion of bacterial contaminants onto meat surfaces or into meat products has prompted concern over the activity and extent of proteolytic action. Similarly, internal proteolysis has served as a cause for much research. Whether the interest lay in explaining the "resolution of rigor" in a carcass or seeking the cause for muscular dystrophy, proteolysis is often put forward as a potential mechanism.

Both types of proteolysis were of concern in this study. Bacterial proteolysis was considered because of the purported role it plays in the "resolution of rigor," the increased tenderness of aged beef, and degradation of quality or functionality. Endogenous proteolysis was considered because of the part it might play in the "resolution of rigor" and in tissue protein turnover.

These studies were undertaken to compare the activity of a Pseudomonas perolens protease with the activity of an endogenous proteolygic enzyme in muscle, the calcium activated sarcoplasmic factor (CASF). During the course of these studies the presence in muscle tissue of another proteolytic enzyme, the kinase activating factor (KAF), necessitated a comparison of these two sarcoplasmic enzymes and the bacterial protease. The activity of these enzymes on various substrates and on myofibrillar integrity was assessed.

These activities were viewed with the possibility of establishing a hypothetical role in protein turnover or tissue protein degradation by the sarcoplasmic proteinases.

II. LITERATURE REVIEW

Sources of Proteases

Bacterial Proteases

Proteolytic enzymes isolated from various microbial sources have long been an area of academic and economic interest. The advent of the commercial market of ficin and papain as important meat tenderizer proteases led naturally to the development of other plant and microbial sources as commercial preparations (Kang and Warner, 1974).

In the area of meat science much debate has ensued as to the cause of the "resolution of rigor," whether it is the result of endogenous enzymes, such as the calcium activated sarcoplasmic factor (Penny et al., 1974; Goll et al., 1974), or of bacterial enzymes.

The bacterial enzymes have been implicated because of their ubiquity and because the most common bacterial contaminant of refrigerated meat, Pseudomonas, has a demonstrated propensity for producing proteolytic enzymes.

The proteases produced by microbial sources have been generally classified as either alkaline, acid or neutral proteases. This classification is dependent upon the assessment of the pH optimum of the isolated enzyme, although some recent work has demonstrated that the pH optimum may also be dependent upon the substrate (Klapper et al., 1973).

The alkaline and acidic proteases have been isolated from various species of microbes and characterized by a number of investigators. Alkaline proteases have been reported from S. aureus (Arvidson et al., 1973), Ps. aeruginosa (Morihara et al., 1973), Asp. oryzae (Nakadai et al., 1973) and Ps. maltophilia (Boethling, 1975). Bosman (1973) reported on an acidic protease from Asp. niger. These are only a few examples of the extensive work that has been accomplished within these two categories of microbial proteases. However, the extremes of the pH optima make them difficult to consider for potential sources of "rigor resolution."

The neutral proteases have been objects of study for a number of reasons. The primary rationale for study is that the pH optimum is generally between 5.0 and 7.5. The secondary reason is that these proteases invariably are activated by one or more metal ions, e.g., Ca^{++}, Zn^{++}, etc. These attributes readily lend the application of this category of protease to various proteolytic mechanisms requiring a "trigger" at an apparently neutral or physiological pH. A number of examples of this class of protease have been found in the literature. Murayama et al. (1969) isolated the biologically active (proteolytic) metabolite of Ps. fragi and found fragin to be a neutral protease. Morihara et al. (1963) isolated two such proteases from Ps. aeruginosa and labeled them proteinase I and II. Porzio and Pearson (1975) did further elucidating work on the Ps. fragi protease. Within the same bacterial genera Buckley (1972) isolated a neutral protease from Ps. perolens.

While the pseudomonad species are studied by the meat scientist other species of bacteria also produce neutral, metal ion activated proteases. Arvidson et al. (1972) found a complete spectrum of proteases in S. aureus. This species released acid, alkaline and neutral proteases. Arvidson (1973) characterized the neutral protease. Other common food-borne microbes also have been shown to release neutral proteases into the medium. Tani et al. (1971) demonstrated this with B. cereus; Grootegoed et al. (1973) with B. caldolyticus; Uehara et al. (1974) with B. subtilis, Dasgupta and Sugiyama (1972) with Cl. botulinum and Hapchuk (1975) with Cl. perfringens.

Nor are the bacterial species the only producers of neutral, metal activated proteases. Some molds also produce such proteases. Asp. niger and Asp. oryzae have been investigated by Bosman (1973) and Nakrani et al. (1973a and 1973b) and Klapper et al. (1973a), respectively. And the regulation and induction of two extracellular proteases from Neurospora carassa have been studied by Cohen et al. (1975).

The ready production of extracellular proteases especially by species associated with food has been established. The critical question concerning the release/production of these enzymes appears to be, What "triggers" this situation? It was found that for many pseudomonads the presence of Ca^{++} was indispensable for proteinase production (Morihara, 1959a and 1959b). Klapper et al. (1973b) have found the age of the cells and the composition of the culture fluid to be factors. Of the two factors the most influential appeared to

be the composition of the culture medium. This became most apparent by the inverse relationship between the glucose concentration and protease release. Boethling (1975) demonstrated a similar effect with Ps. maltophilia. In fact, this author suggested that catabolite repression was the mechanism that was functioning as some form of post-transcriptional control. Alpha-ketoglutarate was found to suppress exoenzyme secretion preferentially with respect to total protein synthesis.

Klapper et al. (1973) and Obdezalik and Chaloupka (1971) found that dense populations in fresh medium produced dramatic increases in protease production for Asp. oryzae and B. megatherium KM Sp^-, respectively.

While all of the above enzymes dealt with extracellular proteases, Johnson (1974) was concerned with the intracellular proteases of S. pneumoniae. His findings corroborated the preceding reports in determining that the extracellular enzymes are indeed inducible; however, he found that intracellular enzymes were constitutive. This was evidenced by the non-reduction of intracellular protease levels when the cells were given a peptide free medium. The extracellular enzyme levels decreased under this regimen.

Proteolytic Activity of Bacterial Proteases

Most of the investigations into the activity of bacterial proteases upon muscle tissue have been macroscopic or chemical in nature. Very little has been done to visualize the results of

bacterial protease activity upon individual myofibrils with sodium dodecyl sulfate gel electrophoresis, or similar techniques.

Of course, the early workers were concerned with identifying the protein group, if any, which was attacked by bacterial proteases within muscle tissue. Price et al. (1962) injected Plasmocid, 6 methoxy-8(3-diethylaminopylamino) quinoline into rats and then examined the diaphragm muscle microscopically. They found severe Z-line degradation as well as actin filament disintegration 18 hours after injection. The sarcotubule system was also destroyed. Observations after 40 hours revealed still further disintegration of Z-line and actin filaments. Disintegration reached a maximum at 72 hours with disoriented I-bands and no Z-discs yet the A-band remained in normal register. Surprisingly, at 120 hours regeneration began to occur. D'Agostini (1963), working with the same system, witnessed the same results but also observed myosin breakdown at 8-10 hours with complete disintegration after 24 hours.

However, Jay (1964) demonstrated that psychrophilic flora, Pseudomonas and Achromobacter, did not break down beef muscle proteins except under exceptionally heavy loads, although significant changes did occur in the sarcoplasmic proteins. In a subsequent paper (Jay, 1969) this activity was attributed to the release of catheptic enzymes by bacterial action. Further support for the utilization of low molecular weight sarcoplasmic proteins was furnished by Jay and Kontou (1967) and Luke et al. (1967). Ockerman et al. (1969) found changes in sarcoplasmic and NPN fractions of sterile beef muscle, but also noted myofibrillar changes. Rampton

(1970) working with hamburger found no change in myofibrillar proteins. These conclusions were further supported in kind by the work of Ingram and Dainty (1971) with fish muscle and red meat.

Borton et al. (1970) found an increase in sarcoplasmic proteins and NPN accompanied by a decrease in myofibrillar proteins. Tarrant et al. (1971) found considerable protein breakdown after 20 days of storage in innoculated sterile pork muscle. The myofibrillar protein in the innoculated pork decreased to one-third of its initial value; however, the sarcoplasmic protein level did not increase or change significantly. The transmission electron microscope corroboration of Tarrant's work was supplied by Dutson et al. (1971). The myofibrils were extremely disrupted in the A-band region, an H-zone devoid of material; a few thick filaments (myosin) were apparent and most of the dense material from the Z-line had been lost. The actin filaments were fairly distinct and in register.

To further confuse the picture of muscle proteolysis, Kang and Warner (1974) demonstrated, using three proteases isolated from papaya latex, that it was the myofibrillar proteins which are more susceptible to digestion than the sarcoplasmic proteins. Very little work has been done with bacterial proteases and individual muscle proteins. However, Morita and Yasui (1973) digested myosin with a calcium activated, neutral protease of bacterial origin. The myosin was digested into constituent fragments: Heavy meromyosin (HMM), Light meromyosin (LMM) and a sub-fragment (S1). They found that the extent of HMM + S1 produced was proportional to myosin:

enzyme ratio and digestion time. Similar results were reported earlier by Kominz et al. (1965) using papain. Ba'lint et al. (1975), also using papain, performed a more sophisticated elucidation of the myosin degradation products. In addition to the above reported results they found that light chain-2 disappeared early in digestion. Fast muscle HMM-S1 yielded 89,000 to 79,000 dalton subunits, further digestion yielded a 50,000 dalton subunit. Slow skeletal muscle and cardiac muscle exhibited greater stability to transformation.

Endogenous Muscle (Vertebrate) Proteases--Digestive Proteases

A number of vertebrate proteases have been studied which until recent isolations were all involved with the digestive or systemic systems. Some of these proteases were trypsin from bovine pancreas (Northrup et al., 1948); chymotrypsin from bovine pancreas (Keith et al., 1947); pepsin from porcine gastric mucosa (Northrup, 1930); thrombin from blood (Schmidt, 1872) and pancreatic elastase from ox pancreas (Walchii, 1878). These enzymes differ from the bacterial proteases in a number of features. All of the vertebrate proteases listed above are serine proteases (i.e., inhibited by diisopropylphosphoro fluoridate, DPF) as opposed to the neutral metal activated proteinases from some bacterial cells. All of the vertebrate proteases listed had acidic optimum pH's, as opposed to neutral pH optima for the bacterial proteases. Perhaps the greatest difference between the bacterial and vertebrate proteases is that the vertebrate proteases are released by their cells of origin as

precursors and need some sort of mechanism to cleave the precursor to release the active enzyme.

The mechanism for the release of the active segment from the precursor involved cleavage either autocatalytically or through some other protease. Trypsin is produced from trypsinogen by limited proteolysis at pH 8 by small amounts of trypsin itself (McDonald and Kunitz, 1941), enterokinase activation (Yamashima, 1958) or thrombin proteolysis (Engel et al., 1966). Chymotrypsin is activated by a more complicated series of steps. Chymotrypsinogen is converted to π-chymotrypsinogen by trypsin proteolysis then by a series of autodegradative steps to α-chymotrypsin (Wright et al., 1968; Corey et al., 1965). The enzyme pepsin is derived from pepsinogen by autocatalysis below pH 5.0 (Vanakis and Herriott, 1956). Thrombin is also activated by a complicated sequential process that proceeds autocatalytically from a calcium activated step (Manusson, 1958). And, finally, proelastase releases elastase by the cleavage of the precursor with trypsin (Uram and Lamy, 1969).

Some of these enzymes, namely, trypsin (Goll et al., 1969; Austin et al., 1974; Syrovy, 1968) and thrombin (Murzbek and Laki, 1974) have been used extensively to study the structural makeup of other proteins by the subunits released from controlled proteolysis. However, these proteases have found limited use in explaining protein turnover or "rigor resolution." A study by Dedman et al. (1975) demonstrated that the binding of aldolase to actin increases its susceptibility to proteolytic attack by trypsin and chymotrypsin.

Intracellular Proteases (Vertebrates)

Other enzymes isolated from vertebrate sources have been implicated more deeply with protein turnover or degradation than those enzymes with various digestive or systemic functions. These enzymes are the lysosomal or catheptic enzymes.

The lysosomal cathepsins have been fairly well characterized as cathepsins A, B1, B2, C, D and E as well as dipeptidyl aminopeptidases I and II (McDonald et al., 1971). The early stage of lysosome study involved discovering the presence of these particles in various tissues. Once the lysosomes were located many attempts were made to implicate them in protein degradation or turnover (Fell and Dingle, 1963; Schwartz and Buchanan, 1967; Stagni and De Bernard, 1968; Davies et al., 1973; Harikumer et al., 1974). A number of investigators found lysosomal activity in muscle (Schwartz and Buchanan, 1967; Stagni and De Bernard, 1968; Harikumer et al., 1974). The muscle studies involved more specific hydrolases than the catheptic enzymes (e.g., acid phosphatase, β-glucouronidase, β-galactosidase, etc.). The next phase of lysosomal study involved isolation of the enzymes from these sacs or organelles and the characterization of these enzymes.

Lysosomes and Cathepsins

With the discovery of the lysosomes by DeDuve (DeDuve and Gianetto, 1955; DeDuve and Wattiaux, 1960) these organelles have undergone intensive research. The enzymes isolated from the lysosomes have been studied because they play an important part in the

degradation of intracellular proteins (Katunuma, 1974). A number of catheptic enzymes have been isolated from the lysosomes. They are cathepsin A (E.C. 3.4.2._), B (E.C. 3.4.4._), C (E.C. 3.4.4.9), D (E.C. 3.4.4.2.3) and E (E.C. 3.4.4._).

The result of extensive study of these enzymes has disclosed a few similarities but many dissimilarities. All of the catheptic enzymes have pH optima in the acidic range (e.g., pH 2-5). However, even this generalization must be tempered by the fact that the pH optima for a particular catheptic enzyme depends upon the substrate. Also, all of the catheptic enzymes have demonstrated proteolytic activity, thus their implication in intracellular protein degradation (Barret and Dingle, 1971).

Even though cathepsins A and C are both classified by activity as exopeptidases their requirements and mode of activity are very different. Cathepsin A does not require the presence of thiols for activity but it cannot act alone, either. Cathepsin A works synergistically with the endopeptidase cathepsin D; without the presence of cathepsin D there is little or no proteolytic activity. Cathepsin C, conversely, can function alone but requires the presence of thiols and Cl^-.

Among the endopeptidases there is a similar lack of uniformity. Cathepsin B requires thiol for activation but has been isolated in two forms, cathepsin B1 and B2. Cathepsin B1 has a molecular weight of 24,000 daltons and hydrolyzes benzoyl arginine-p-nitroanilide (BAPA) or benzoylarginine-1-naphthylamide (BANA) (Greenbaum, 1971). To further confuse possible classification,

when attempts are made to separate these two enzymes by Sephadex chromatography (G-100) they elute as a single protein peak with two peaks of activity. Yet when this protein peak is placed on a DEAE-Cellulose-52 column and eluted, 4 to 5 components with B1 activity are eluted and 2 components are isolated with B2 activity (Franklin and Metrione, 1972). Furthermore, cathepsin B1 possesses protease activity, whereas B2 does not exhibit protease activity (Franklin and Metrione, 1972). Cathepsin D and E are distinguished from each other on the basis of their respective abilities to attack different substrates and different pH optima. Cathepsin E has a molecular weight of 305,000 daltons, whereas cathepsin D has a molecular weight of 58,000 daltons (Barret and Dingle, 1971). It has been reported that at low temperatures cathepsin E may be converted to cathepsin D (Greenbaum, 1971).

Much work has been reported on localization studies for lysosomal enzymes (catheptic enzymes). These enzymes have been investigated for their role in biology as well as their influence on food products. Bodwell and Pearson (1963) presented some of the properties of the catheptic enzymes in relation to beef muscle. They found their preparation activated by 1 mM $FeCl_2$ and inhibited by 1 mM iodoacetate. Thus they were probably assaying cathepsins B and D. Their studies on the muscle components actomyosin, myosin and actin revealed that it probably was the assay conditions, pH 4.4 and 37°C that assisted the lability of these proteins, and protein degradation may not have been the result of the catheptic enzymes. Similar studies were attempted with other species such as the rabbit

(Suzuki and Fujimaki, 1968) and fish and chicken (Fukushima et al., 1971). However, later investigators (Eino and Stanley, 1973a and 1973b; Eitenmiller, 1974) concentrated on trying to isolate cathepsin D specifically because of its activity as a protease. At this time the chicken became the species most preferred for lysosomal study because of its characteristic of increasing lysosomes during starvation. The lysosome content would be increased by starvation; however, the question remained whether the lysozyme content (catheptic enzymes) distribution was normal. This question prompted studies on the location and specific activity of the catheptic enzymes in chicken (Caldwell and Grosjean, 1971) and fish (Reddi et al., 1972). However, most of the studies were inconclusive. This especially seemed true for centrifugation studies, probably because of the difference in particle size (Reddi et al., 1972).

Aside from the question of catheptic localization, the concern over catheptic involvement in protein turnover prompted further studies. The relationship of the catheptic enzymes in normal and dystrophic muscle became the subject of study. The results of these investigations are exemplified by the work of Iodice et al. (1972). They worked with chickens from the 18-day embryo to 1 month after hatching. Cathepsin A activity was high in both normal and dystrophic chickens initially, then declined at a linear rate in the normal chicken but remained the same in the dystrophic chicken after hatching. Cathepsin B increased in the dystrophic chicken and apparently in the increased activity accounted for the total increase in autolytic activity. Cathepsins C and D increased significantly

2 weeks after hatching. The level of cathepsin C in the dystrophic chicken was 6 times the level in the normal chicken. The result of all of this analysis led the authors (Iodice et al., 1972) to conclude that cathepsin B was the controlling enzyme in protein degradation. Similar findings were reported for denervated frogs (Krishnamoorty, 1971).

Based on the conclusions of previously cited papers a number of investigators (Eino and Stanley, 1973; Eitenmiller, 1971; Moeller et al., 1976) probed the effect of catheptic enzymes upon various muscles and structural components. Pre-rigor intact muscle fibers that were treated with a crude cathepsin preparation were degraded and fragmented as viewed by the scanning electron microscope (Eino and Stanley, 1973b). The above mentioned study also noted changes in the tensile properties of muscle, such as a decrease in breaking strength and breaking elongation. These same investigators examined catheptic activity in postmortem beef muscle. They found that hydrolytic activity (catheptic activity) affected the sarcoplasmic proteins most. Then in decreasing severity the proteins from the endoplasmic reticulum, myofibrillar proteins and the stromal proteins were affected (Eino and Stanley, 1973a). As for the major protein components of muscle, actin, myosin and actomyosin, there was no detectable enzymatic activity or induced change. This is supported by the work of Bodwell and Pearson (1963) but conflicts with report of sarcoplasmic protein susceptibility being the highest with myosin A, actin and myosin B following in decreasing susceptibility to proteolysis (Suzuki et al., 1969). Eino and Stanley

(1973a) also found that cathepsin D was mainly responsible for the pattern of tenderization in beef psoas major muscle. Because of the implication of catheptic enzyme activity in muscles, both antemortem and postmortem, there followed concern over the effect of various processing treatments upon the catheptic enzymes. It was found that the temperature of cure for country-cured hams would not affect cathepsin D, but that salt concentrations greater than 0.5 M, increasing sucrose concentrations and concentrations of potassium nitrate greater than 0.01 M could affect cathepsin D activity (Deng and Lillard, 1973). Pre-treating country-style hams with cathepsins increased free amino acid production (Melo et al., 1974). This interest carried over into other flesh foods with the discovery that during storage of shrimp muscle (Penaeus setifereis) cathepsin D activity was discovered but not cathepsins A, B or C (Eitenmiller, 1974).

The greatest stumbling block in assessing the contribution and/or presence of the catheptic enzymes is in the determination of whether the hydrolytic activity is the result of cathepsins and, if so, which one. This prompted a number of studies utilizing various techniques to separate and identify catheptic activity (DeLumen and Tappel, 1972; Taylor et al., 1974; Baykowski and Frankfater, 1975).

Other Types of Cellular Proteinases

Investigations into catheptic activity and its relation to lysosomes and protein turnover led to the discovery and identification of other types of cellular proteases.

Studies on the degradation of the muscle of a common Taiwan fish (Ophicephalus tadiana) led to the isolation of a protease in macerated liver with a pH optimum of 9.0, however, no activity was found in the muscle (Utzino and Hishiwaki, 1951). Considerable proteolytic activity was found in rat liver nuclei (Dounce and Umana, 1962). Apparently a number of proteases are at work since peaks of maximum activity were found at pH 3.0, 3.5, 4.5, 7.0 and 9.0. Generally, the activity increased with increased damage to organelles.

Working with other organs and cell types, Moore et al. (1970) isolated proteases from erythrocytic membranes; however, the enzymes may be located in the interior of the erythrocyte. Proteases from erythrocytes may be bound to a lipoprotein and resemble chymotrypsin. Similarly, a protease was isolated from blood platelets (Legrand et al., 1973). Commercial myoglobin preparations from horse skeletal muscle demonstrated an ability to degrade casein (Goldspink, 1971). The protease from the myoglobin preparation also demonstrated an ability to attack the proteins in rat myofibrils and was inhibited by EDTA and p-chloromercuribenzoate (PCMB). Such studies prompted the comparison of rat mast cell granule protease and a bovine capsule protease for similarities of activity despite different origins (Lloyd et al., 1971). A proteinase isolated from rabbit acrosomes was found to resemble human trypsin in activity (Stambaugh and Smith, 1974). Oftentimes the investigators were not specifically looking for a protease when the activity was encountered in the enzyme preparations. The discovery of proteolytic activity

in bovine milk xanthine oxidase preparations is an example of this type of discovery (Nathans and Hade, 1975).

Proteases have been found in cells with a well-defined lineage as those used for cell culture. Earlier investigations found that protease inhibitors added to the culture medium yielded transformed cells, as well as decreased the amount of cell proliferation (Schnebli and Burgler, 1972). This situation may have been resolved by the discovery of cell surface proteases which may be important regulators of cell growth and cell-cell interactions (Grinnell, 1975).

The inability to totally attribute "rigor resolution" to bacterial proteolysis prompted the search for endogenous proteolytic enzymes in skeletal muscle. Aside from the catheptic enzymes, which have already been mentioned, a number of proteolytic enzymes have been isolated from muscle tissue (Busch et al., 1972; Huston and Krebs, 1968).

A protease was isolated from rat skeletal muscle supernatant which had a pH optimum between 8.5 and 9.0; it was activated by 1 mM $FeCl_3$ but not inhibited by iodoacetate. This enzyme was inhibited by PCMB (Kozalka and Miller, 1960). This may be the enzyme isolated by Goldspink et al. (1971) from horse skeletal muscle myoglobin preparations. Similarly, an alkaline protease was isolated from muscle by Holmes et al. (1967). Proteolytic activity was reported for a crude homogenate of the myofibrils of rat skeletal muscle. Activity was greatest for the myofibrillar fraction as compared among

crude homogenate and other fractions, including the supernatant (Noguchi and Kandatsu, 1966).

A study on the endogenous proteolysis that results during storage of rabbit muscle disclosed that 82% of the degradation products were of low molecular weight equivalents (e.g., anserine, etc.). After 7 days storage at 3-4°C the high molecular weight fraction of muscle decreased from 12% to 8% and the low molecular weight fraction increased to 90% (Suzuki et al., 1967). Thus, as disclosed earlier, there is some endogenous proteolysis. In an unrelated study the effect of proteolysis upon the emulsification property of bovine skeletal muscle was investigated and it was found that apparently a mean optimum size of protein fragment is reached (a point of high emulsification) then surpassed (Dubois et al., 1972).

At that time proteolytic enzymes were implicated in activities other than the proteolytic breakdown of muscle tissue. A proteolytic enzyme with a neutral pH optimum and activated by 1 mM Ca^{++} was implicated in the activation of skeletal muscle phosphorylase kinase (Krebs and Huston, 1968). Interestingly, Goldspink et al. (1971) theorized that the protease which they isolated was this particular enzyme although they offered no proof.

In an investigation that followed up the controversy as to where the muscle protease was located, supernatant (Kozalka and Miller, 1960) or the myofibrillar fraction (Noguchi and Kandatsu, 1966), it was found that the supernatant or sarcoplasmic fraction possessed the ability to inhibit the proteolytic enzyme (Noguchi and

Kandatsu, 1969). Not only the sarcoplasm but the blood serum from the rat had the ability to inhibit proteolytic activity (Noguchi and Kandatsu, 1969). In a follow-up paper the same authors demonstrated that the proteolytic activity they reported for the myofibrillar fraction is different from catheptic (cathepsin D) activity. In fact, though more than half of the catheptic activity of rat skeletal muscle was recovered in the myofibrillar fraction, the catheptic activity in this fraction can be removed by extraction with dilute saline solution containing Triton X-100 (Noguchi and Kandatsu, 1970). This enzyme had a pH optimum between 7.5 and 9.5 and only diisopropyl-fluorophosphate inhibited the enzyme activity. It was unaffected by EDTA, iodoacetamide and PCMB (Noguchi and Kandatsu, 1971).

While debate continued concerning the location and nature of the alkaline enzyme of skeletal muscle, a proteolytic enzyme was isolated from rabbit skeletal muscle tissue that had a neutral pH optimum (Busch et al., 1972). The enzyme was activated by 1 mM $CaCl_2$ and was found in the sarcoplasm of skeletal muscle. These investigators reported the removal of Z-line material from the myofibrils upon incubation of the enzyme with 5 mM $MgCl_2$ and 1 mM $CaCl_2$. To further complicate the picture, a neutral protease system was isolated from rabbit skeletal muscle. This system consisted of an endopeptidase (protease I) and an exopeptidase (protease II) (both active at pH 7.2). Protease I was activated to 1 mM $CaCl_2$. Protease II yielded ambiguous results in activation studies, however, both proteases were inactivated by iodoacetate and EDTA (Okitani et al., 1974).

In a recent series of papers the relationship between the proteases and muscle tissue breakdown was examined (Banno et al., 1975a, 1975b, 1975c). They demonstrate the presence of group specific proteases. While their study involved denervation experiments they postulated that in muscle the group specific proteases for this tissue may be involved in the control level of phosphorylase in muscle. Could the enzyme isolated by Krebs and Huston (1968) be part of this neutral protease system?

The calcium activated sarcoplasmic factor or protease isolated from skeletal muscle by Busch et al. (1968) was investigated by a number of laboratories. It was found that this protease could affect the integrity of myofibrillar cells by Z-line degradation (Penny, 1974). Of the individual components of the myofibril exposed to this protease, it was found that only tropomyosin, α-actinin and troponin were substrates for it. Actin and actomyosin were not substrates for this protease and it was further suggested that Z-line degradation was the result of activity of this protease upon α-actinin (Penny, 1974). Other investigators found that the release of α-actinin from myofibrils occurred in the absence of detectable change in the electrophoretic profile of the other myofibrillar proteins, whereas trypsin-treated myofibrils yielded extensive degradation of myosin (Reddy et al., 1974).

Metabolic Interconversion of Enzymes

The calcium activated sarcoplasmic factor isolated by Busch et al. (1968) has been investigated for its possible role in "rigor

resolution" or the breakdown of protein intracellularly. However, it is uncertain if this is the true physiological role of this protease or whether there are other functions.

Krebs and Huston (1968) isolated a factor, the kinase activating factor, for which they claimed the ability to activate skeletal muscle phosphorylase kinase. The isolation of this protease explained the activation of phosphorylase kinase by Ca^{++}. Until the isolation of this protease the activation by Ca^{++} of phosphorylase kinase was baffling. Prior to the discovery of this Ca^{++} effect the interconversion of phosphorylase b to a was interpreted as depicted in Figure 1. In this scheme the primary enzyme in the conversion was protein kinase, which was activated by cAMP produced by membrane bound adenyl cyclase. The protein kinase in turn converts inactive phosphorylase b kinase to the active form which in turn converts phosphorylase b to the more active phosphorylase a by phosphorylation of two phosphorylase b subunits with 4 moles of ATP (Fischer and Krebs, 1955; Krebs et al., 1969). The above described sequence applies to the in vivo physiological sequence as determined by a number of investigators. Indeed, much recent work involved studies on the conformational changes suspected in the conversion of the dimeric phosphorylase b subunits to the tetrameric phosphorylase a (Brooks et al., 1973).

In spite of this characterization there appears to be anomalies in the system. One investigation (Heilmeyer et al., 1970) described an event known as "flash activation." "Flash activation" occurs when phosphorylase b and phosphorylase kinase are incubated

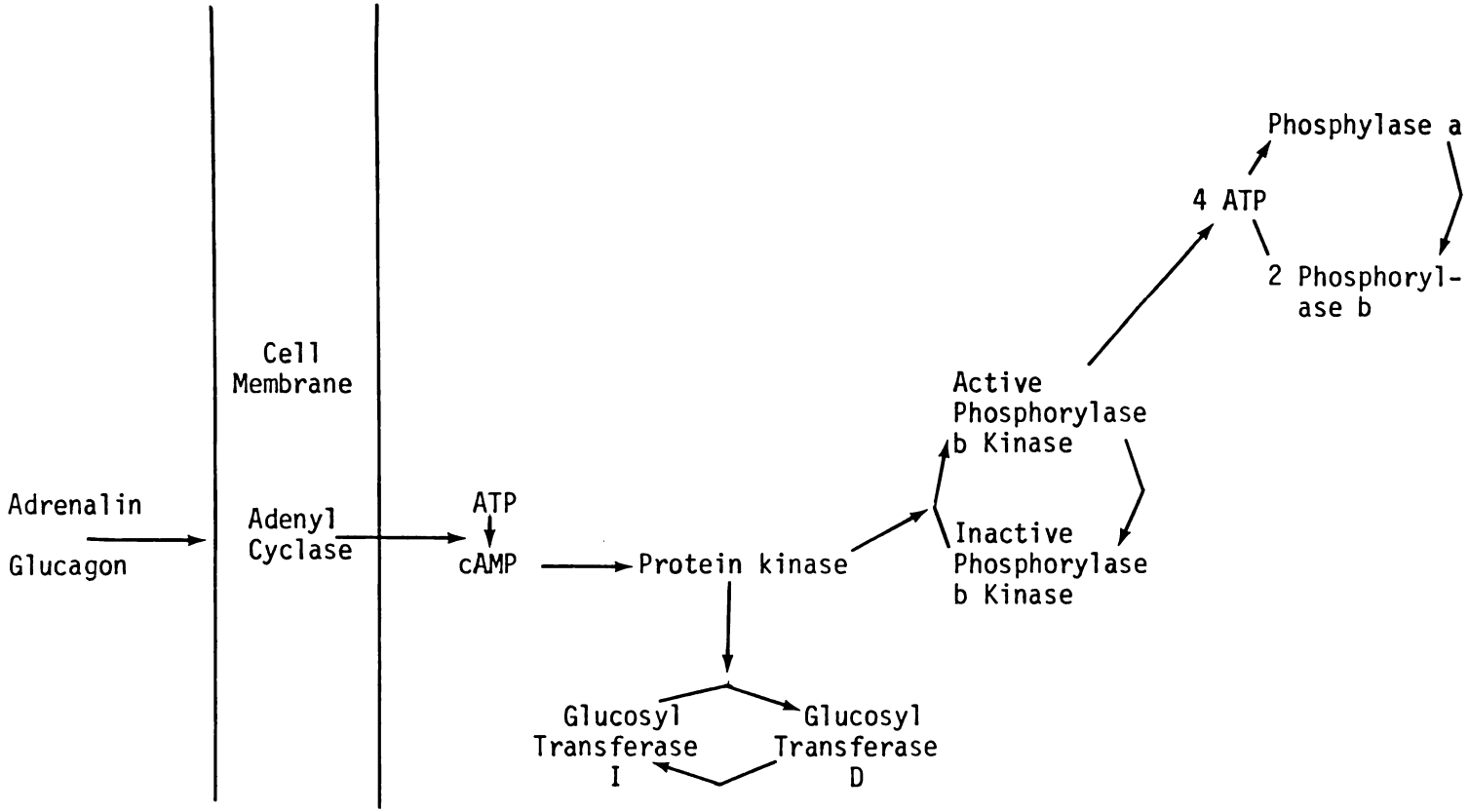


Figure 1.--The control of phosphorylase activity by an enzyme cascade system (adapted from Newsholme and Start, 1973).

with ATP, Mg^{++} and Ca^{++}. Subsequent papers delved into the properties of phosphorylase b kinase and the requirements for its activation. Phosphorylase b kinase isolated from skeletal muscle has little activity but it can be activated in vitro in a number of ways (Newsholme and Start, 1973): (a) incubation with ATP and Mg^{++}; (b) incubation with Ca^{++}; (c) incubation with proteolytic enzyme, trypsin; and (d) increasing pH to 8.2.

The activation of phosphorylase b kinase by a large upward shift of pH is not a very likely physiological event. Activation through Mg^{++} and ATP is thought to reflect the influence of hormonal control upon the enzyme through the system depicted in Figure 1 (Newsholme and Start, 1973). Although the same authors add the proviso that some other factor may be involved in cAMP activation which is removed in purification. However, some authors (Yamamura et al., 1971) postulate a de-repression system for the action of cAMP. However, recent studies imply that cAMP activation may involve dissociation of the regulatory subunit from the active catalytic subunit (Huang and Huang, 1975). These studies apply not only to skeletal muscle but to other tissues such as liver (Yamamura et al., 1971), adipose and cardiac (Corbin et al., 1975; Corbin et al., 1972), and to other species (e.g., rat; Gibson and Newcomb, 1975).

The Ca^{++} activation caused some investigators (Newsholme and Start, 1973) to view this phenomenon as possible control of the enzyme cascade by the nervous system, although Na^{+} and K^{+} were reported to have little effect (Gibson and Newcomb, 1975). However, the need for a intracellular proteolytic enzyme (Krebs and Fischer,

1962; Krebs and Huston, 1968; Meyers et al., 1964) to affect the Ca^{++} activation phenomenon of phosphorylase b kinase casts some doubt as to a physiological role. The high concentration of Ca^{++} needed for activation, 1 mM, was demonstrated by Ozawa et al. (1967) to be the result of trace contaminants. When all traces of Ca^{++} were removed from the system, phosphorylase b kinase was activated by 1 - 0.1 μmole Ca^{++}. This returned the Ca^{++} concentration to physiological limits but did not explain the irreversible role of the protease.

The protease (KAF) functioned by removing the catalytic subunit from the regulatory subunit. Perhaps it also functions during cAMP activation of the enzyme (Huang and Huang, 1975). Cyclic AMP activation and Ca^{++} activation of phosphorylase b kinase were mimicked by trypsin (Huang and Huang, 1975; Krebs and Huston, 1968).

Thus there appears to be another function for an intracellular protease in the cell other than protein turnover. Also, the connection between Ca^{++} activation of the enzyme that activates the enzyme which breaks down glycogen and stops glycogen synthesis, and its relation to skeletal muscle contraction, is intriguing. Further interrelationship between skeletal muscle contracture, protein turnover and glycogen metabolism is demonstrated by the ability of phosphorylase b kinase to phosphorylate troponin, specifically, troponin I (Stall et al., 1972; Penny and Cole, 1974).

Protein Turnover

Because skeletal muscle is the largest single tissue in the mammalian body, around 45% of the body weight, it must have a significant role in metabolism (Young, 1970). This author has further stated that the pool of free amino acids in the muscles of large animals can represent a large part of their daily requirements, thus serving a buffering capacity with respect to amino acid needs. This amino acid pool, as well as the mass of skeletal tissue, is subject to the steady state relationship between protein synthesis and degradation (Munro, 1970; Young, 1970; Goldberg and Odyessey, 1972; Young, 1974). The assessment of this steady state is further complicated by the reutilization of amino acids from the pool (Waterlow, 1969). The extent of this reutilization varies from tissue to tissue and according to nutritional state. In the liver reutilization of amino acids varied according to the nutritional regimen from 50-90% (Young, 1970), whereas muscle reutilization was reported to be 10 to 30% (Waterlow and Stephen, 1968). Reutilization leads to errors in turnover rate measurement. One further consideration must be made in the study of the degradation of individual proteins and their relationship with the total tissue; different size proteins have different degradation rates. Thus, according to Goldberg and Dice (1974), larger proteins possess higher degradation rates than smaller proteins; however, they further state that this might not apply to the myofibrillar proteins. Additionally, the tissue of interest must be studied and not a simple system such as bacterial proteins, since investigators have demonstrated different rates and

mechanisms for mammalian and bacterial systems (Goldberg and Dice, 1974; Goldberg, 1972a).

Much of the substrate for the maintenance of free amino acid pools, especially in the post absorptive state, is thought to be derived from the breakdown of tissue protein (Woodside and Mortimer, 1972). These authors state further that amino acid requirements for protein synthesis, gluconeogenesis and numerous metabolic reactions continue into the post absorptive period. It may therefore be assumed that in the absence of an adequate external supply of amino acids internal sources are made available. The mechanism and regulation of protein degradation is poorly understood. In a study of the proteins of the endoplasmic reticulum, Arias et al. (1969) found them to be in a dynamic state with a mean half life of 2 days, suggesting rapid turnover. This turnover is the result of a trade off between the zero order rate of synthesis and the first order rate of degradation (Schimke, 1970). The protein catabolism of rat liver homogenates was found to be inhibited by EDTA, ATP, ADP and Krebs cycle intermediates (Brostrom and Jeffay, 1970). Goldberg (1972a) found that the degradation of bacterial proteins was enhanced in proteins that incorporated amino acid analogs when compared to non-analog containing protein. He found that proteins which normally turned over rapidly were also the most susceptible to tryptic digestion. Also, proteins with larger subunits were degraded faster than proteins with small subunits (Glass and Doyle, 1972). A small change in a protein will cause its selective breakdown, even though it is undetectable immunologically (Capecchi et al., 1974). Thus,

hypoxanthine-guanine phosphoribosyl transferase (HGPRT) that was defective broke down 3 to 17.5 times faster than normal HGPRT.

Protein turnover in rat liver could be suppressed by 80% with the infusion of an amino acid mixture comparable to ovalbumin hydrolysate but lacking leucine, isoleucine, valine and tyrosine (Woodside and Mortimore, 1972). In muscle, Bullock et al. (1972) found that for the glucocorticoids, corticosterone possessed the greatest ability to decrease weight gain in the whole animal and in the vastus lateralis, vastus medialis and gluteus medius muscles. Insulin was found to prevent the block in peptide chain initiation and thus decrease protein turnover through its influence upon the steady state in the direction of synthesis (Jefferson et al., 1974; Fulks et al., 1975; Goldberg, 1972b). Woodside et al. (1974) demonstrated that glucagon stimulated proteolysis and inhibited biosynthesis. The introduction of insulin and glucose reduced the amino acids released from rat diaphragms as measured through tyrosine which is not degraded or synthesized in the muscle; however, the amount of alanine released was not reduced (Fulks et al., 1975). Using the longissimus and hind limb muscles of suckling piglets, the half lives for synthesis and degradation were measured for sarcoplasmic and myofibrillar proteins (Perry, 1974). The rate of synthesis and degradation for sarcoplasmic proteins was 4.8 and 9.4 days, respectively. The values for the myofibrillar proteins were 5.7 and 16.4 days, respectively. With a model system of isolated rat heptaocytes, it was found that ammonia reduces nitrogen loss by affecting the rate of protein degradation (Seglen, 1975).

Aside from the study of protein degradation and turnover in normal skeletal muscle, liver and other systems, many studies have concerned themselves with the effect of an inadequate diet, fasting and starvation. A number of early studies demonstrated that during prolonged protein-calorie malnutrition muscle loses a greater percentage of its initial protein content than does liver (Young, 1970). The proteins of the contractile fibers diminish while extracellular proteins (e.g., collagen) are not reduced. During 144 hours of starvation Bucko et al. (1968) measured the amylase, lipase and protease activity of rat pancreatic homogenates. They found that enzymatic activity decreased least in protease function. Lipase and amylase activity showed the greatest reduction. Noguchi and Kandatsu (1969) in a study monitoring the autolytic activity of rat muscle proteins demonstrated that the sarcoplasm and blood serum inhibited myofibrillar autolytic activity. A follow-up study demonstrated that this autolytic activity differed from catheptic activity (Noguchi and Kandatsu, 1970). Millward (1970a, 1970b) found that the half lives for synthesis of liver, sarcoplasmic and myofibrillar proteins were 1.0, 2.8 and 7.2 days, respectively. Whereas, for catabolism in the same tissues, half lives were 1.8, 3.6 and 15.6 days, respectively. With a protein free regimen the catabolic rate of liver protein increased by 20% with a small increase for myofibrillar proteins, also. After 3 days of starvation the liver demonstrated only a slight increase in catabolism, whereas there was a 75% increase in the rate of myofibrillar breakdown.

Alanine and glutamine were determined to be the major amino acids released by muscle during fasting. Since alanine comprises only 10% of the muscle amino acids, the synthesis of alanine from pyruvate was postulated by Felig and Wahren (1974). Stansbie et al. (1975) demonstrated similar effects upon pyruvate dehydrogenase activity through alloxan diabetes and starvation. Both conditions greatly decreased the phosphorylated form of the enzyme, however, this control mechanism is restricted to adipose tissue. A similar study with adipose lactate dehyrogenase isozymes also demonstrated a similar effect between starvation and diabetes. They also found that for malnourished rats the growth rate appeared to be more efficient than for well fed rats, 41% and 22%, respectively. They also found the myofibrillar protein turnover to be non-random.

The similarity in response of diabetic and starved rats with respect to protein turnover prompted the investigation of muscular abnormalities. Zalkin et al. (1962) studied the effects of vitamin E deficient diet induced muscular dystrophy and lysosomal enzymes and activity. They found that ribonuclease, cathepsin, β-galactosidase and aryl sulfatase all increased after four weeks of deficiency. Furthermore, enzyme activity increase correlated with histopathological changes. In a review by Young (1970) some pertinent points were made concerning dystrophic muscles, e.g., synthesis of muscle protein was maintained or increased, ribosomal activity was higher, RNA content was higher but degradation also increased. In summary, the loss of protein appeared to result from increased degradation. The work of Kitchen and Watts (1973) supports the hypothesis that

there is no obvious defect in the protein synthetic machinery of dystrophic muscle but that certain proteins showed anomalous turnover patterns relative to normal animals. Shafig et al. (1972) demonstrated that Duchenne type muscular dystrophy is much more degradative in nature than in the model of study, chicken muscular dystrophy; thus demonstrating the error of the model system approach. Ingwall et al. (1975) proposed fetal rat hearts as a model system of ischemia. Using this system he demonstrated an increase of lysosomal activity (β-acetyl glucoseaminidase, cathepsin D and acid phosphatase) with increased length of ischemia. A contrary event was reported by Baraona et al. (1975) in a study involving the long-term feeding of ethanol to rats. They reported the accumulation of protein in the soluble cell fraction of the liver suggested decreased degradation.

All of the research cited above illustrates that protein turnover, and especially the degradation rate of specific proteins, myofibrillar in particular, are subject to a fine adjustment with the nutritional state of the entire animal.

III. EXPERIMENTAL METHODS

Bacterial Propagation and Enrichment

A stock culture of Pseudomonas perolens ATCC 10757 was obtained from the American Type Culture Collection and stored in a cooler at 4°C. Culture propagation was performed as follows: The sealed outer vial was removed by breaking, using heat and water to cause the glass to fracture. The pure culture contained in the inner vial in lyphilized form was dissolved in 0.5 ml sterile water. This solution was transferred aseptically to a screw cap test tube containing 10 ml of sterile nutrient broth (Difco Manual). Incubation was carried out at room temperature (25°C) for 36 hours at which time growth was indicated by turbidity of the solution. Stock cultures for subsequent use were then prepared by innoculating test tubes containing 10 ml of sterile nutrient broth with two loopfuls of the initial solution and allowed to incubate at room temperature until slight turbidity was observed. These were then stored in a freezer at -23°C until required.

Cultures used for experimentation were kept at room temperature in nutrient broth (10 ml) in screw cap test tubes. Growth was kept vigorous by daily transfers of one loop of medium from the growing tube to a fresh, sterile tube of nutrient broth. To further aid in maintaining the homogeneity of the culture, the stock culture that was stored in the freezer was thawed at room temperature, then

two loops of the thawed culture were transferred to fresh, sterile nutrient media (10 ml). These tubes were incubated at room temperature. The growth resulting from this transfer continued to be propagated at room temperature with daily transfers.

The homogeneity of the culture was checked periodically by microscopic observation, gram stain and growth in litmus milk medium (Breed et al., 1957).

Enrichment

One loop of Pseudomonas perolens ATCC 10757 cultivated at room temperature in nutrient broth was transferred to a 500 ml Erlenmeyer flask containing 125 ml of nutrient broth. The flask was placed in a water bath shaker (Eberbach) at room temperature and shaken moderately for 24 hours. The subsequent growth medium was transferred to a plastic centrifuge bottle (250 ml) and centrifuged at 12,000 x g for 20 minutes on the RC2B centrifuge (Sorvall). The pellet was slowly redissolved in 5 ml 0.1 M Tris-HCl, 0.0045 M $CaCl_2$ (pH 7.5). A 2% (2.5 ml) innoculum of the redissolved pellet ($O.D._{660}$ + 1.5) was added to another 500 ml Erlenmeyer flask containing 125 ml of nutrient broth and cultivated in the shaker water bath in the above described manner. Twenty-four hours later, the centrifugation and redissolving of the pellet were repeated. After the pellet had been redissolved in 25 ml 0.1 M Tris-HCl, 0.0045 M $CaCl_2$ (pH 7.5) buffer, the mouth of the centrifuge tube was flamed and the contents of the centrifuge bottle were emptied into a 2 liter Erlenmeyer flask. The flask contained 500 ml of Casamino Acid Medium

(Difco) with metal salts added ($ZnCl_2$ and $CaCl_2$). The optical density of the redissolved pellet was approximately 2.2-2.4. The innoculated 2 liter Erlenmeyer flasks were flamed and stoppered with cotton and then placed on a shaker table in a 10°C cooler. The cultures were shaken at this temperature for 60 hours. If proper innoculation and cultivation took place then the medium changed from a whitish-grey color to a green, frothy medium.

Media Preparation

All media were prepared using deionized, double, distilled water. Absorbent cotton and aluminum foil were used as stopper and cover, respectively, during sterilization which was carried out at 15 psi, 121°C for 15 minutes. The sterile medium was allowed to cool to room temperature prior to innoculation.

Casamino acid medium (Difco) was chosen as the medium for bacterial growth and enzyme production. Its composition was as follows:

Casamino acid medium	4.51 grams
$CaCl_2$	0.51 grams
$ZnCl_2$	0.014 grams

The medium was cultured at 10°C and always received a 2% innoculum.

Protein Extraction

Sarcoplasmic Protein

A modification of the method by Helander (1957) was used. Five grams of muscle was placed in 40 ml of 0.05 M phosphate buffer (pH 7.5) in a Waring Blendor. The muscle/buffer mixture was subjected to two 15-second bursts, and then poured into 250 ml plastic

centrifuge bottles. The blendor was rinsed with 10 ml of the 0.05 M phosphate buffer (pH 7.5) and extracted for 3 hours. After centrifugation and filtering through cheese cloth the volume of sarcoplasmic protein extracted from five grams of muscle was recorded. Fifteen milliliter samples in duplicate were used to determine the quantity of water soluble protein (sarcoplasmic) present.

Myofibrillar Protein

The pellet from the sarcoplasmic protein extraction was further extracted with 50 ml 1.1 M potassium iodide for 3 hours at 2-6°C. This mixture was centrifuged for 20 minutes at 1,400 x g and filtered through 8 layers of cheesecloth. The residue was resuspended and centrifuged. The combined volume of the supernatants was recorded. Fifteen milliliter samples in duplicate were used to determine the quantity of myofibrillar protein present in a five gram sample of muscle.

Non-protein Nitrogen

This fraction was determined by adding 5 ml of 10% TCA (w/w) to 15 ml of the sarcoplasmic protein extraction supernatant. After addition of the 10% TCA the solution was shaken, then allowed to stand for 2-4 hours at 2-6°C. After standing the solution was centrifuged at 12,000 x g for 20 minutes on the RC2B centrifuge. The supernatant was decanted into Kjeldahl flasks for the nitrogen analysis.

Total Protein Nitrogen

Approximately 0.5 g of muscle was weighed on nitrogen free paper and subjected to semi micro Kjeldahl analysis (American Instrument Co.).

Stroma Protein

This value was obtained by subtracting the values of the sarcoplasmic, myofibrillar and NPN fractions from the total nitrogen per gram value.

Protein Determination

Kjeldahl

The micro Kjeldahl method as proposed by the American Instrument Company (1961) was used.

Fluorescamine Protein Determination

For samples that were in the microgram per milliliter quantity the fluorescamine assay was used. To 0.5 ml of sample was added 1.5 ml 0.2 M sodium borate (0.2 M boric acid titrated with NaOH) buffer (pH 9.25). The tube was swirled and 0.5 ml of fluram reagent [4-phenylspiro(furan-2|3H|,1'-phtalan)-3,3'-dione] was added. Fluorescence was read on an Aminco-Bowman Spectrofluorometer with the excitation wavelength set at 390 nm and the emission wavelength set at 480 nm and the percent transmission was recorded. Bovine serum albumin was used to set up a standard curve from 1 µg to 100 µg/ml (Appendix A.1).

Lowry Method of Protein Determination

For enzymatic analyses and most protein assays the Lowry method (1951) was used in a modified manner. To 1 ml of protein solution or dilution was added 5 ml of Lowry solution C (Appendix A.2); the tube was shaken and incubated for 20 minutes at room temperature. To the above mixture was added 0.5 ml phenol reagent (Folin-Ciocalteau phenol diluted 1:1 with water) and the tube was allowed to stand for 45 minutes at room temperature with occasional mixing. The optical density was read at 660 nm with a Beckman Model 24 Spectrophotometer. Bovine serum albumin was used to set up a standard curve from 20 μg/ml to 500 μg/ml.

Biuret Method of Protein Determination

The concentration of myofibrillar solutions was determined via the biuret method. The method was used as outlined in A.O.A.C. (1965).

Blood Serum Studies

In order to ascertain various physiological and biochemical changes occurring in the adult male rabbits during *ad libitum* feeding and an extended (28 day) fast, blood was collected, prepared and assayed at regular intervals.

Blood Serum Collection

After each rabbit was weighed, the ear of the rabbit was cleaned with 95% ethanol (v/v with water). The ear vein was then

located and a small puncture was made with the tip of a scalpel or razor blade. At the first sign of bleeding the ear was placed into a glass suction device designed for rabbit ear bleeding, nine inches of vacuum was applied and the blood collected in a polyethylene centrifuge tube located at the bottom of this device. Approximately 7-10 ml of blood were collected at each bleeding; the tube was capped and placed on ice. The ear was treated with 1:1000 Thimersol tincture (Eli Lilly and Co.). The ears were alternated for bleeding to assist healing. The blood samples were allowed to stand at room temperature for 2-3 hours. After standing the blood was loosened from the walls of the tube and cooled at 4°C for 24 hours. Serum was separated by centrifugation at 10,000 rpm in a Sorvall RC2B Centrifuge, decanted into 7 dram plastic snap cap vials and frozen at -30°C. Prior to use the samples were thawed at 10°C.

Serum Total Free Amino Acids

The vial containing the serum was inverted several times (3) before using for the assay. The sample (serum) was diluted 1:4 with double, distilled deionized water and 0.2 ml of the diluted serum was added to the experimental tube. To the diluted serum was added 1.6 ml 0.05 M sodium borate [($Na_2B_4O_7 \cdot 10H_2O$) 19.07 g/l (pH 9.2)]. The mixture was stirred and 0.2 ml 0.25% trinitro benzene sulfonic acid (0.25 g/100 ml water) was added and mixed. This mixture was incubated at 37°C for 20 minutes. After incubation 2 ml 1 N HCl was added to each tube, and it was stirred before the O.D. was read at 420 nm on a Beckman Model 24 Spectrophotometer. Citrulline was used

as a standard. The stock solution of citrulline (0.3504 g/200 ml water) was diluted to a range 0.2 μm/ml (0.2 mM) to 4.0 μm/ml (4.0 mM). This method was adapted from Palmer and Peters (1969).

Serum Glucose Assay

When the vials were thawed to prepare serum for the total free amino acid assay duplicate samples were also taken for the glucose assay. To a standard 10 ml test tube was added 0.02 ml of serum, or deionized water (blank) or glucose solution standard (20-400 m%). To each tube was added 5.0 ml buffered enzyme dye solution. This solution consisted of 100 mM phosphate buffer (pH 7.0). 0.8 units peroxidase/ml (E.C. 1.11.1.7), 10 units glucose oxidase/ml (E.C. 1.1.3.4) and 1.8 mM 2,2'azinodiethylbenzthiaxoline sulfonic acid. The tubes were mixed and allowed to stand at room temperature for 30 minutes. After standing the mixture was transferred to a cuvette and absorbance at 600 nm was read on a Beckman spectrophotometer. All results were reocrded and reported as mg% glucose per milliliter.

Nonesterified Free Fatty Acids

Into a 12 x 75 mm dispo culture tube was pipetted 0.2 ml of serum and the tubes were placed on ice. One milliliter of Dole extract mixture (40 volumes isopropanol, 10 volumes heptane and 1 volume 1N H_2SO_4) was added to the tube, vortexed and allowed to stand for 10 minutes. After standing 0.2 ml heptane and 0.2 ml distilled water were added, the tubes were vortexed and placed on ice for 5-10 minutes. From this point through the rest of the assay

only 12 tubes were handled at a time. The assay was set up to carry 10 sample tubes and 2 blanks through to completion as one unit.

After the phases had separated 0.2 ml of the heptane layer (top) was removed and placed in a 1.5 ml polypropylene centrifuge tube (Brinkman micro test tubes). To each centrifuge tube was added 0.1 ml of the ^{63}Ni solution (Into 100 ml of 1 M triethanolamine (14.92 g/100 ml add 0.4050 g $NiCl_2.6H_2O$, dissolve and then added 0.65 ml $^{63}NiCl_2$. The nickel chloride concentration was approximately 1 mg/ml and the specific activity of ^{63}Ni was 10^6 cpm/100 µl] and 0.8 ml chloroform. The tubes were capped and vortexed well for 45 seconds. The tubes were centrifuged for 10 minutes at 500 x g in a Sorvall RC3 Refrigerated Centrifuge. After centrifugation the tubes were removed from the centrifuge and placed on ice to cool. Care was taken to avoid disturbing the layers. The ^{63}Ni layer was aspirated off with a Pasteur pipette with a finely drawn tip. A 0.5 ml aliquot was placed in a scintillation vial. The chloroform was allowed to evaporate before adding 10 ml of scintillation fluid. This fluid consisted of 160 g naphthalene, 10 g PPO (2,5 diphenyloxazole), 0.1 g dimethyl POPOP (1,4-Bis-2-[4-methyl-5-phenyloxazolyl]-benzene), 770 ml xylene, 770 ml 1,4 dioxane and 460 ml absolute ethanol. Each vial was counted for 10 minutes in a scintillation counter. Results were reported as nm FFA/ml.

A standard curve was determined by dissolving 0.0256 g palmitic acid in 100 ml absolute ethanol (10^{-3} M); then a 1:10 dilution using the previous solution was prepared with 100 ml absolute ethanol. The ethanol was dried off and 0.2 ml of water was added to

each tube (serum volume). After this step the samples were treated as prescribed by the procedure at the addition of Dole extract mixture. Standards were 10-100 nm/ml FFA. This technique was adapted from Ho (1970) as modified by Bieber (1974) and cited by Carstairs (1975).

Enzyme Isolation

Isolation of Pseudomonas Perolens Protease

After the supplemented Casamino acid medium was innoculated with Ps. perolens ATCC 10757 it was cultured for 60 hours. After 60 hours the medium was added to 250 ml polypropylene centrifuge bottles. The medium was centrifuged at 8,000 x g for 20 minutes on the RC2B Sorvall Refrigerated Centrifuge. After centrifugation the pH of the retained supernatant was adjusted to 7.0 with 1 N NaOH.

DEAE-Sephadex A-50 which had been equilibrated in 0.1 M Tris-HCl, 0.0045 M $CaCl_2$ (pH 7.0) was slowly added to the supernatant. The DEAE-Sephadex A-50 was added at a ratio of 5.5 g of equilibrated DEAE-Sephadex A-50 per 25 ml of supernatant. This mixture was stirred slowly and gently with a glass rod and then allowed to stand for 20 minutes. After the DEAE-Sephadex-Enzyme mixture was gently stirred once again to achieve an equal distribution, the mixture was poured into an air-aspirator attached to a Buchner Funnel fitted with a Whatman No. 41 filter paper insert. The supernatant collected in the flask was discarded and the flask replaced under the Buchner Funnel. The DEAE-Sephadex A-50 was eluted with 0.8 M NaCl, 0.1 M Tris-Hcl, 0.0045 M $CaCl_2$ (pH 8.0), to

give approximately 150 ml of eluent per liter of original supernatant. The eluent was retained. $(NH_4)_2SO_4$ was added to the eluent to 50% saturation. The ammonium sulfate was added slowly at 4°C and at the completion of the ammonium sulfate addition the mixture was allowed to stand for 10 minutes. The mixture was centrifuged at 10,400 x g for 20 minutes and the supernatant was discarded. The pellet was dissolved in 0.01 M Tris-HCl, 0.0045 M $CaCl_2$ (pH 7.5) and dialyzed against this buffer for 24 hours.

The protein solution was placed on a 2.5 x 45 cm column of Sephadex G-100, equilibrated with 0.1 M Tris-HCl (pH 7.5) and 5 ml fractions were collected at a flow rate of 15 m./hour. Protein and enzyme activity were monitored by assays described in this section. The active fractions were pooled and stored.

Isolation of Calcium Activated Sarcoplasmic Factor

All procedures were carried out at 4°C. Rabbit back and hind leg muscles were homogenized in a Waring Blendor in 2.5 volumes of 0.004 M EDTA (pH 7.6) for 1 minute and then centrifuged at 14,000 x g for 20 minutes. The pH of the supernatant was then adjusted to pH 6.1 with 1 N acetic acid and allowed to stand for 20 minutes. The resulting precipitate was removed by centrifugation at 14,000 x g for 20 minutes. The supernatant was further acidified to pH 4.9 with 1 N acetic acid and left to stand in ice for 10 minutes. The precipitate was collected by centrifugation at 10,000 x g for 15 minutes and the pellet suspended in 0.1 M Tris-Acetate, 0.004 M EDTA (pH 8.0). Seventy milliliters of buffer was used per kilogram of original

muscle tissue. After the pH of the suspension was adjusted to 7.0 with 1 N KOH the volume of the extract was adjusted to 200 ml/kg of original muscle with cold, double-distilled deionized water and clarified by centrifugation at 20,000 x g for 2 hours. The clarified supernatant was precipitated by ammonium sulfate fractionation. The fractions precipitated between 20-40% saturation (w/v) and at 40% saturation were retained. The precipitates were separated by centrifugation at 10,000 x g for 25 minutes and were resuspended in 0.1 M Tris-Acetate, 0.002 M EGTA, 0.001 thioglycollic acid and 0.001 M NaN_3 (pH 7.5). The ammonium sulfate fractions were adsorbed to a 2.5 x 45 cm DE 52 cellulose column equilibrated with the buffer used to resuspend the pellets. The column was eluted with a linear gradient of 0.0-0.8 M KCl in the same buffer. The active fractions were pooled and precipitated with 40% ammonium sulfate (w/v) and chromatographed on a 2.5 x 45 cm column of Sephadex G-200 equilibrated with 0.005 M glycerophosphate, 0.001 M thioglycollic acid and 0.002 M EGTA (pH 7.5). The active fractions were pooled and concentrated by ultrafiltration and stored at -20°C. This procedure was adapted from Goll et al. (1974) and Reddy et al. (1975).

Isolation of Kinase Activating Factor

The hind leg and back muscles of a rabbit were removed, chilled in ice and ground in a meat grinder. The ground muscle was homogenized in a Waring Blendor in 2.5 volumes (l/kg) of 0.004 M EDTA (pH 7.0) at 4°C for 1 minute. The homogenate was centrifuged for 40 minutes at 4,000 x g. The supernatant was decanted through

glass wool previously washed with double-distilled, deionized water. The pH of the supernatant was adjusted to 6.1-6.2 with 1 N glacial acetic acid and centrifuged for 30 minutes at 4,000 x g after standing for 5-10 minutes. The sediment was discarded and the supernatant pH was adjusted to 4.9-5.0 with 1 N acetic acid and centrifuged for 20 minutes at 5,000 x g. The precipitate was resuspended in 70 ml of 0.1 M sodium glycerophosphate, 0.004 M EDTA (pH 8.2), per kilogram of original muscle weight.

The frozen material from 10 kg of muscle was thawed and diluted to 140 ml/kg of muscle with cold, double-distilled, deionized water. The diluted suspension was centrifuged at 78,000 x g for 2 hours and the supernatant was retained. The mixture was added to a 2.5 x 45 cm DEAE-cellulose column equilibrated with 0.05 M sodium glycerophosphate, 0.002 M EDTA (pH 7.0) with a flow rate of 100-200 ml/hr. The column was washed with one liter 0.1 M sodium glycerophosphate, 0.002 M EDTA (pH 7.0). The kinase activating factor was eluted with 0.3 M sodium glycerophosphate, 0.002 M EDTA (pH 7.0) and collected in 25 ml fractions. The combined fractions with the highest activity were dialyzed against 30 volumes 0.002 M EDTA (pH 7.0), and then concentrated three times with ultrafiltration. The concentrate was further dialyzed against two 1-liter portions of 0.01 M sodium glycerophosphate, 0.002 M EDTA (pH 6.5). After dialysis the fractions were diluted to 4 mg/ml with 6.5 pH buffer. Three successive portions of Alumina C_γ were used as follows: (1) 0.3 mg Alumina C was added per milligram of protein, (2) the mixture was homogenized by hand, (3) stirred for

15 minutes and (4) centrifuged for 5 minutes at 12,000 x g after each addition of gel. Each portion of gel was then homogenized carefully with 15 ml 0.05 M sodium glycerophosphate 0.002 M EDTA (pH 7.0), stirred and centrifuged as before. The alumina C_2 product was adjusted to 1.8 M ammonium sulfate by the addition of 0.9 volumes of 3.75 M ammonium sulfate, stirred at 0°C for 20-30 minutes and centrifuged 5 minutes at 25,000 x g. The precipitate was then dissolved in 4 ml of 0.05 M sodium glycerophosphate, 0.002 M EDTA, 0.5 M NaCl (pH 7.0) and then applied to a 2.5 x 45 cm column of Sephadex G-200 equilibrated with the same buffer. The elution rate was 6-12 ml/hr. The fractions with the highest KAF activity were pooled and dialyzed overnight against 1 liter of 0.05 M sodium glycerophosphate, 0.002 M EDTA (pH 7.0).

Isolation of Inhibitory Factor for Kinase Activating Factor

The neutralized supernatant remaining after the precipitation of the kinase activating factor (KAF) at pH 5.1 was adjusted to pH 5.4 by the addition of 2 N HCl. The adjusted supernatant was placed in a stainless steel beaker and brought rapidly to a temperature of 50°C in a water bath and kept at this temperature for 5 minutes. After rapid cooling (-20°C blast freezer), the denatured protein was removed by centrifugation 4,000 x g for 20 minutes at 0°C and the solution was adjusted to pH 6.8. Solid ammonium sulfate was added to 60% saturation while the pH was kept at 6.8 by the addition of 1 N KOH. After stirring for 10 minutes the mixture was centrifuged as before. The supernatant was discarded and the

precipitate was dissolved in 0.050 M sodium glycerophosphate, 0.002 M EDTA (pH 7.0). The dissolved precipitate was thoroughly dissolved against several changes of the above buffer.

The resulting solution was brought to pH 5.0 with 1 N acetic acid. While stirring, 1.7 ml of calcium phosphate gel (20 mg/ml) was added to produce a gel-to-protein ratio of 0.1. After 10 minutes the suspension was centrifuged, 5,000 x g for 20 minutes. To the supernatant fluid, 2.3 ml of calcium phosphate gel was added, as before, to produce a gel-to-protein ratio of 0.5. After centrifugation the supernatant was discarded and the three gel precipitates were combined and washed by stirring with 4 ml of water followed by centrifugation. The inhibitory factor was then eluted from the gel by stirring for 30 minutes at 4°C with 5 ml of 0.2 M glycerophosphate, 0.25 M potassium chloride and 0.002 M EDTA (pH 7.0). Following centrifugation the gel was re-extracted 4 ml and then with 6 ml of the same buffer. The combined eluates were dialyzed overnight against 0.20 M glycerophosphate, 0.001 M EDTA (pH 7.0). The resulting solution was placed in dialysis casing and concentrated to 2.5 ml. (Method was modified from Drummond and Duncan, 1966.)

Calcium Phosphate Gel Preparation

To 9.1 g of KH_2PO_4 was added 33 ml of 1 N HCl and the mixture was warmed until it dissolved. After cooling to room temperature, 14.7 g of $CaCl_2.H_2O$ was added and the solution diluted to a final volume of 50 ml with deionized, distilled water. The solution was added to 41 g of cellulose powder (Whatman CF 1) in 200 ml of

water. The mixture was stirred rapidly for no more than 2 minutes and 55 ml of 8 N NH_4OH was added. Stirring was continued for 10 minutes. The pH was 9.0. The slurry should become thick upon standing overnight at 10°C. The supernatant was decanted and two lots of gel cellulose were combined. The combined gel-cellulose was washed by decantation of 3 liters of water until the supernatant was negative to the Nessler reagent. Fines were removed. The gel-cellulose was collected by low speed centrifugation and resuspended in 1 liter of appropriate buffer or water. The gel-cellulose could be stored at either room temperature or at 5°C.

Enzyme Activity Assays

Calcium Activated Sarcoplasmic Factor Activity

The proteolytic activity was assessed upon casein substrate. To a reaction mixture of 0.4 ml of 0.05 M Tris-Maleate buffer (pH 6.9), 0.1 ml 0.005 M $CaCl_2$ or EGTA and 0.5 ml 5.0 mg casein was added 0.16 mg CASF. Incubation time of 60 minutes was allowed and the reaction was terminated by the addition of 1 ml of 10% cold trichloroacetic acid. The mixture was allowed to sit in ice for 10 minutes and was then centrifuged. Aromatic amino acids released were measured by the method of Lowry (1951) and expressed as µg of tyrosine equivalents. Control and blank samples as well as standards were run with every assay as modified by Busch et al. (1972).

Kinase Activating Factor Activity

KAF fractions to be assayed were diluted in 0.05 M Tris-HCl, 0.001 M EDTA, 0.045 M 2-mercaptoethanol buffer (pH 7.5), containing 0.5 mg/ml BSA (Bovine serum albumin). Prior to the activity test all fractions were incubated for 1 hour at 30°C. To 0.2 ml of the diluted KAF was added 0.2 ml of nonactivated phosphorylase kinase (E.C. 2.7.1.38) solution in the same buffer at a concentration of 10,000 units/ml as assayed at pH 8.2. To this mixture was added 0.2 ml of 0.09 M Tris-HCl, 0.03 M calcium acetate (pH 7.5), and the solution was incubated for 5 minutes at 30°C. The reaction was stopped by the addition of cold neutral 0.015 M cysteine to a 1:30 dilution. The phosphorylase kinase activity was then assayed at pH 6.2 as follows: The initial reaction mixture consisted of 0.2 ml 0.125 M Tris, 0.125 M glycerophosphate at pH 6.2, to which was added, 0.2 ml AMP-free phosphorylase b (E.C. 2.4.1.1) solution (8.7 mg/ml crystalline phosphorylase b/ml) in 0.015 M neutral cysteine, 5 minutes before the assay. The assay was initiated by the addition of 0.1 ml of the activated phsophorylase kinase in cold, neutral 0.015 M cysteine. The mixture was placed in a water bath at 30°C and after 5 minutes of incubation time the reaction was stopped by transferring a 0.1 ml aliquot in duplicate to 1.8 ml of 0.04 M glycerophosphate, 0.03 M cysteine buffer (pH 6.2), at room temperature. To further assay for phosphorylase a (E.C. 2.4.1.1), to 0.2 ml of 0.032 M glucose-1-phosphate, 2% glycogen solution was added 0.2 ml of the diluted kinase reaction mixture. The solution was allowed to incubate for 5 minutes at 30°C, and the reaction was

stopped by the addition of 8.2 ml of a solution containing 0.5 meq H_2SO_4 and 25 mg ammonium molybdate. To 0.3 ml of this final solution was added 1.2 ml of isobutanol-benzene (1:1) and 1.2 ml of acid molybdate (1.5 g of ammonium molybdate in 100 ml of 0.5 N H_2SO_4). The tube was shaken for 45 seconds, the phases allowed to separate and the upper layer was read at 410 nm in 1 ml cuvettes. Each μ mole of free phosphate gave 0.023 O.D. units above the blank, thus Beer's law was obeyed. (The method was a modification of the procedure of Huston and Krebs, 1968.)

Catheptic Assay

The assay for cathepsin D used the hemoglobin digestion method of Anson (1938) as modified through the addition of 0.002 M $FeCl_2$ by Bodwell and Pearson (1963).

Proteolytic Assay

The caseinolytic assay used by Buckley (1972) was modified by the substitution of 0.03 M Tris-HCl (pH 7.5) for the phosphate buffer.

Enzyme Kinetics

To ascertain the basic enzyme kinetic parameters for *Pseudomonas perolens* protease the method of Morihara *et al.* (1969), Morihara and Tsuzuki (1971) and Morihara (1974) was used.

The reaction mixture contained 0.04 M Tris-acetate buffer, 0.0045 M $CaCl_2$, 0.004 M suitable peptide and 0.5 ml enzyme in a 3 ml system with a pH of 7.5. The peptides consisted of N-carbobenzoxy-L-

alanyl-L-leucine (Sigma Chemical Co.) and N-carbobenzoxy-glycyl-L-leucine (Sigma Chemical Co.), initially. The substrate system was equilibrated in a water bath for 180 seconds with shaking prior to introduction of the protease. The amount of enzyme added to the system was 2 units, 3 μg/system. After 10 minutes of reaction a 0.2 ml aliquot was placed in 1.0 ml reagent to begin the assay and stop the reaction. This reagent consisted of 0.2 M citrate, 0.01 M EDTA, 0.4 M NaOH (pH 5.0). To this mixture was added 1.2 ml KCN-methylcellosolve-ninhydrin. This mixture was heated for 15 minutes at 100°C, cooled in running tap water for 5 minutes and then diluted to 3.0 ml with 60% ethanol (v/v with water). The entire assay mix was added to a cuvette and its optical density at 570 nm recorded. The method used was Yemm and Cocking (1955) as modified.

For the kinetic study varying concentrations of substrate were added and the K_m and V_{max} determined according to Mahler and Cordes (1971) and Gutfreund (1972).

Myofibril Studies

Myofibril Preparation

Adult male rabbits were sacrificed by exsanguination and the back muscle was immediately excised and suspended in a relaxing buffer consisting of 100 mM KCl, 10 mM Tris-acetate (pH 7.00), 2 mM $MgCl_2$, 2 mM EGTA, 10 mM NaN_3, 0.2 mM DTT and 2 mM $Na_4P_2O_7$. The procedure for myofibril preparation was that of Etlinger et al. (1973) with the following modifications: phenylmethylsulfonyl fluoride (25 mg/L) was added to the homogenizing buffer, and the

0.02% sodium desoxycholate extraction was replaced by a 0.1% Triton X-100 extraction.

Phase Contrast Microscopy

The technique of Busch et al. (1972) was used. Samples were viewed and photographed with a Zeiss Photomicroscope III.

Sodium Dodecyl Sulfate Polyacrylamide Gel Electrophoresis

SDS gel electrophoresis was carried out according to Weber and Osborn (1969) as modified by Porzio and Pearson (1976).

Prior to SDS gel electrophoresis the sample was dialyzed overnight in 0.5% SDS, 0.05 M Tris-glycine (pH 8.8), 0.5% 2-mercaptoethanol. Approximately 8 ml of sample and 1 ml 2.5% SDS were added to the dialysis bag, or some proportion thereof. The sample was dialyzed overnight and prior to application to the gel was incubated for half an hour at 37°C. The sample was applied to the gel upon addition of concentrated glycerol to 10-20% and 1-2 drops Pyronin Y (50 µg/ml) tracking dye.

The gel solution consisted of the following: 10 ml acrylamide [2.5 g acrylamide (Biorad) and 0.025 g Bis (Biorad, N,N'-Methylene-bis-acrylamide]; 5 ml gel buffer (0.5 M Tris, 1.5 M glycine diluted 1:10, pH 8.8); 2.5 ml glycerol (reagent grade, 50% with H_2O); 1.0 ml SDS; 1.0 ml TEMED (1% solution N,N,N',N' tetramethylenediamine); 4.5 ml H_2O and 1.0 ml 1% ammonium persulfate. The TEMED and ammonium persulfate were added last to the mixture, prior to addition to the tubes. The above mixture should fill

twelve 5 x 100 mm gel tubes. The tubes were layered with water and allowed to polymerize. Before placing the gel tubes in the chamber buffer they were flushed out with this buffer.

The chamber buffer consisted of 0.2 M Tris-glycine, 0.1% SDS (pH 8.8) diluted 1:10.

The sample was added to the gels and the gels were electrophoresed for 6-10 hours at 0.5 mA/tube.

At the completion of the run the gels were removed from the gel tubes and fixed in 25% (w/v) isopropanol, 10% glacial acetic acid for 1-3 hours. The gels were stained overnight with 0.002% Coomassie blue (w/v) in 50% methanol (v/v) and 10% glacial acetic acid.

IV. RESULTS AND DISCUSSION

Development of Pseudomonas Perolens ATCC 10757 Protease

Growth and Enrichment of Pseudomonas Perolens

Buckley (1972) isolated a protease from Ps. perolens ATCC 10757 and characterized it. In the medium of his choice, Koser's Citrate supplemented with calcium chloride (0.5 g/l), maximum growth was achieved at 60 hours with enzyme production detected at 47 hours. Calcium was found to be required for enzyme production as Morihara (1959a) postulated for extracellular enzyme production in the pseudomonads. The efficacy of this medium is demonstrated in Figure 2. Thus the medium used supported growth and the extracellular protease was produced. However, it was decided to attempt to improve enzyme production by various enrichment procedures. The improved enzyme production was necessary if the method of isolation described by Buckley (1972) was to be used. His isolation procedure involved an 80% loss of enzyme activity in the first isolation step.

Innoculum Density

Prior to the development of a new medium the ability of an enriched innoculum to increase enzyme production was tested. By allowing the cells to multiply to dense populations through cultivation in nutrient broth at room temperature with shaking for twelve hours, a very dense young cell innoculum was obtained. To assure

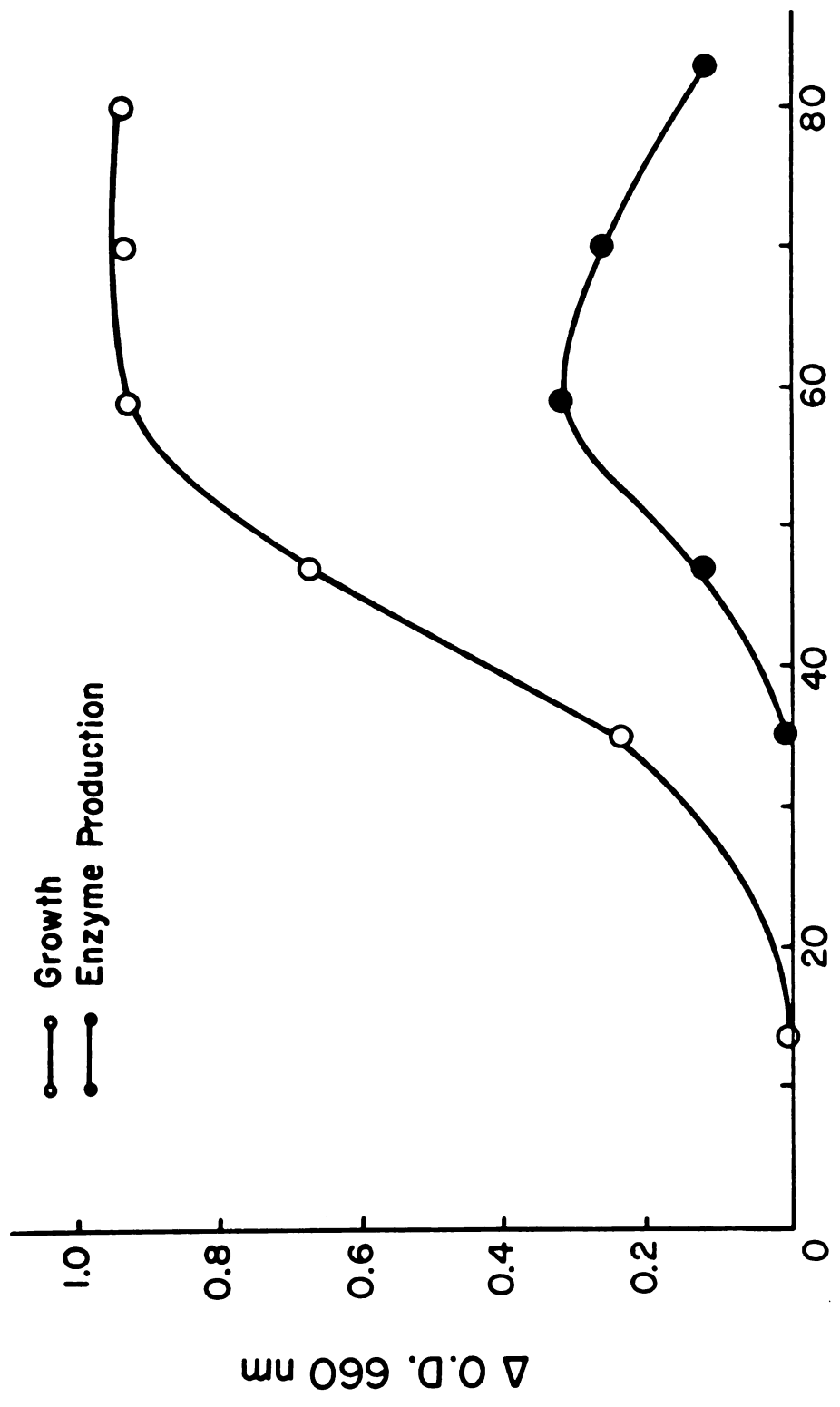


Figure 2.--Growth of Pseudomonas perolens ATCC 10757 and enzyme production in Koser's citrate medium plus 0.5 g/l calcium chloride, pH 7.5, at 10°C. (Adapted from Buckley, 1972.)

maximum culture density the density of the final innoculum was increased through two transfers in enriching media prior to innoculation in the growth flask. Each transfer increased the density of the innoculum while maintaining the cell population young and vigorous (log phase). This density technique yielded rapidly growing and vigorous cultures. This work confirmed the observations of Obdezalik and Chaloupka (1971) and anticipated the results of Klapper et al. (1973b). They found that dense populations in fresh medium produced dramatic increases in microbial growth and enzyme production. This increase in growth and enzyme production due to innoculum density is apparent in Table 1. By increasing the innoculum density fourfold, the specific activity of the enzyme obtained was increased 223%. All subsequent experiments used this technique of high innoculum density.

Medium Enrichment

Tarrant et al. (1971) used a complex mixture of eleven amino acids and two dipeptides with essential metal salts for the cultivation of Pseudomonas fragi ATCC 4973. Buckley (1972) found that this medium did induce enzyme production by Pseudomonas perolens ATCC 10757 but that greater enzyme production resulted from the Koser's citrate supplemented with 0.5 g/l calcium chloride. The medium suggested by Tarrant et al. (1971) did yield greater bacterial growth than the supplemented Koser's citrate.

According to Johnson (1974) and other investigators, the extracellular enzymes were inducible, whereas the intracellular

TABLE 1.--Effect of innoculum density upon the production of protease by Pseudomonas perolens ATCC 10757 in Koser's citrate with 4.5 mM $CaCl_2$ (pH 7.5) grown at 10°C.

Innoculum	Supernatant		Total Protein (mg)	Enzyme Activity		Specific Activity
	Volume (ml)	µg/ml		µg Tyr/ml/min	Total	
Normal	250	9.0	2.25	0.1	25	11.1
Dense	470	14.1	6.63	0.6	282	42.5

enzymes were constitutive. Enzyme production may be induced by either positive feedback or metabolite de-repression (Davis et al., (1967). Koser's citrate medium contained citrate as a carbon source and sodium ammonium phosphate as a nitrogen source. It was decided, therefore, to substitute a new carbon source for citrate. D-glucose in the same concentration as citrate (1.2×10^{-2} M) and at twice the concentration (2.3×10^{-2} M) was used. Furthermore, the carbon and nitrogen source were combined into a single molecule, ammonium citrate, at the original citrate concentration. The results of this study are summarized in Table 2 (see also Appendices B.1.1 and B.1.2). It is apparent from Table 2 that all of the substituted media had greater protein yields in the supernatant than the unsubstituted Koser's citrate medium. The medium with glucose substituted for citrate at the same concentration yielded 249% more

TABLE 2.--Effect of substituted carbon and nitrogen sources upon the ability of Pseudomonas perolens ATCC 10757 to produce an extracellular protease in Koser's citrate medium (pH 7.5) at 10°C.

Substituted Source	Supernatant Volume (ml)	Average		Specific Activity (units/mg)
		Total Protein (mg)	Total Activity (units)	
Control	220	6.7	48.4	7.2
Glucose	220	16.7	677.0	40.5
Glucose, 2x	230	29.2	--	--
Ammonium citrate	220	12.9	2,176.0	168.7

protein than the control medium. Doubling the glucose concentration yielded 175% greater protein over the glucose medium and 436% more protein than the control medium. For the substituted media, the medium with the substituted nitrogen source had the poorest yield of only 193% above the control. However, the ammonium citrate medium demonstrated the greatest amount of enzyme activity over all of the media in the test. Glucose added at the same concentration as citrate exhibited a protease with 14 times more activity than the control. The double glucose concentration medium demonstrated no measurable enzyme activity. It would seem that the presence of the large excess of carbon source precluded the need for protease production. The greatest specific activity was evident in the ammonium citrate medium. Studies comparing Koser's citrate and ammonium citrate directly yielded similar results.

It became apparent in the previous studies that some form of inducer was necessary for the production of protease. Therefore, the ability of a protein (Hammerstein's casein) and a hydrolyzed casein mixture (Casamino acids) to induce protease production was determined. These media were attempted even though Griffin and Fogarty (1973a, 1973b) met with little success in cultivating the production of a metallo-protease from Bacillus polymyxa. A further assessment in this experiment involved the need for zinc in the medium. It has been found that a number of neutral proteases were stabilized by a metal ion (Ca^{++}) and activated by another metal (Zn, Mn, etc.), according to Conn et al. (1964). Buckley (1972) attempted to use $ZnCl_2$ in Koser's citrate but substituted it in place of

the $CaCl_2$. In spite of this he reported slow growth but no enzyme production.

The casamino acid medium yielded greater protein accumulation than either the protein basal medium or the Koser's citrate medium. This was evidenced by Figure 3 (see also Appendix B.2.2). The protein basal medium did not produce any large amount of protein, although most of the studies demonstrated an anomalous rise at 60 hours only to return to a normal level.

The addition of 0.01 mM $ZnCl_2$ to the medium resulted in a dramatic increase in protein yield. The yield from the casamino acid medium more than doubled. While the increases for the Koser's citrate and protein basal medium were not as dramatic, they also increased in yield. (See Figure 4 and Appendix B.2.3.)

Not only was growth enhanced by the addition of 0.01 mM $ZnCl_2$ but enzyme production almost doubled (see Figure 5). For the casamino acid medium the enzyme activity doubled through the addition of $ZnCl_2$. Similar increases were noted for the Koser's citrate medium although the activity did not double. However, the protein basal medium responded to Zn^{++} addition by halving its enzyme activity. The effect of Zn^{++} upon protein and enzyme production was apparent in this study.

Apparently Zn^{++} is necessary for the production of enzyme by Pseudomonas perolens ATCC 10757. However, Zn^{++} in quantities greater than 10^{-3} M may actually have a toxic effect upon the bacterial cell. In a separate study it was found that 10^{-5} M Zn^{++} was the optimal concentration for enzyme production (Appendix B.2.4).

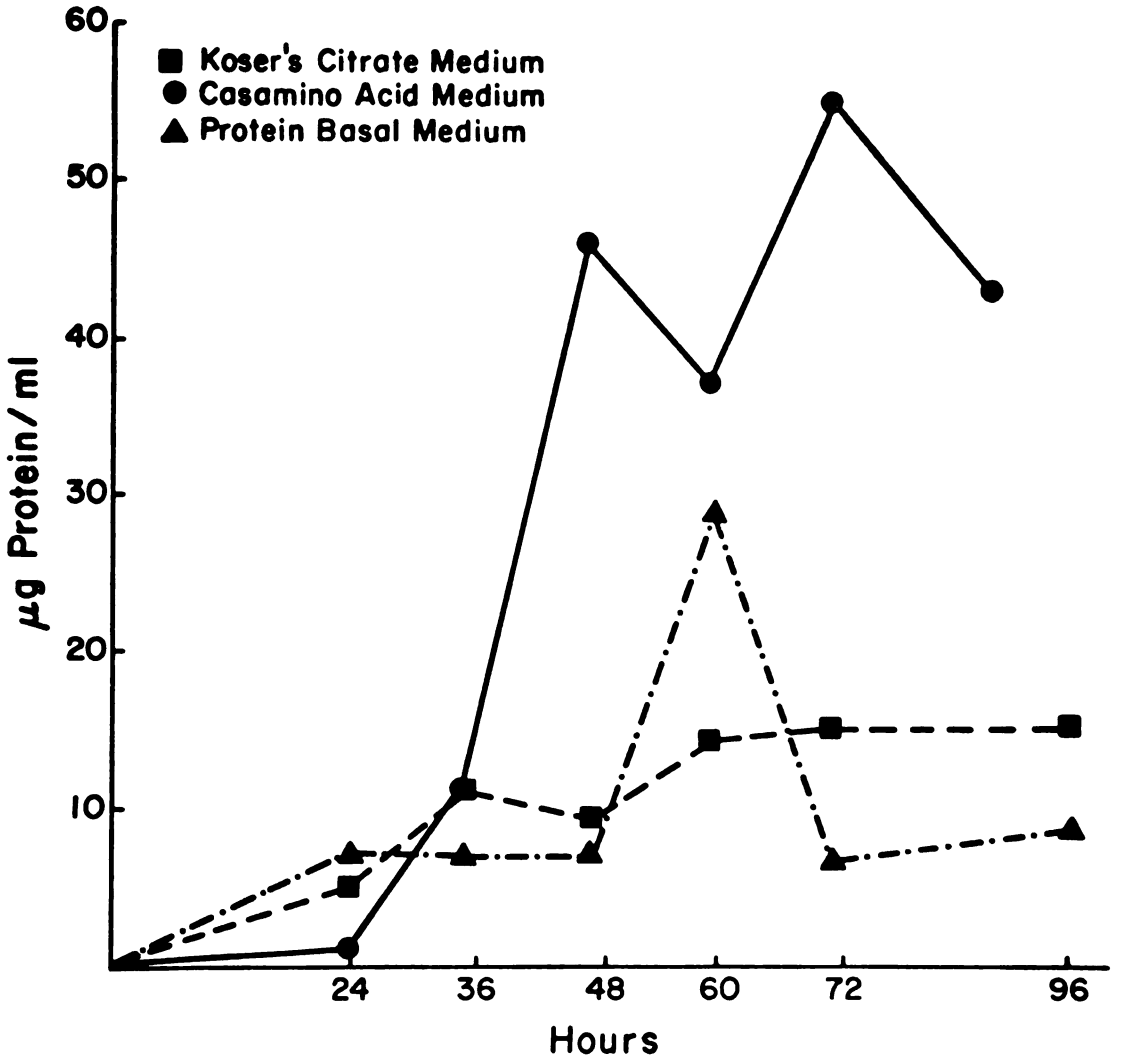


Figure 3.--The effect of the addition of protein or protein hydrolysates upon the growth of Pseudomonas perolens ATCC 10757 in various media at pH 7.5 and 10°C. All media contained 4.5 mM $CaCl_2$.

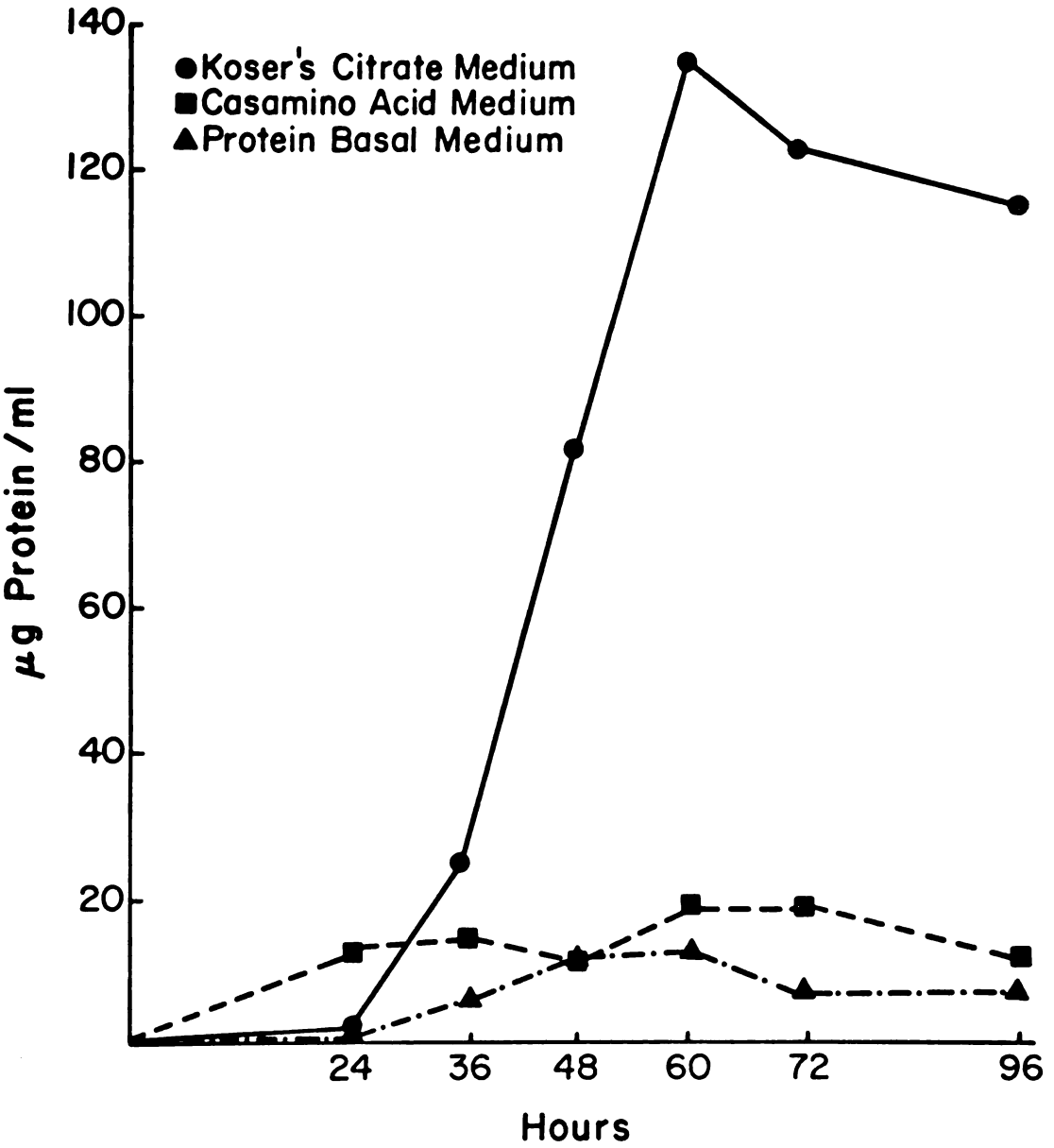


Figure 4.--The effect upon protein production by Pseudomonas perolens ATCC 10757 by the addition of $ZnCl_2$ (10^{-5}) M to various growth media cultured at pH 7.5 and 10°C. All media contained 4.5 mM $CaCl_2$.

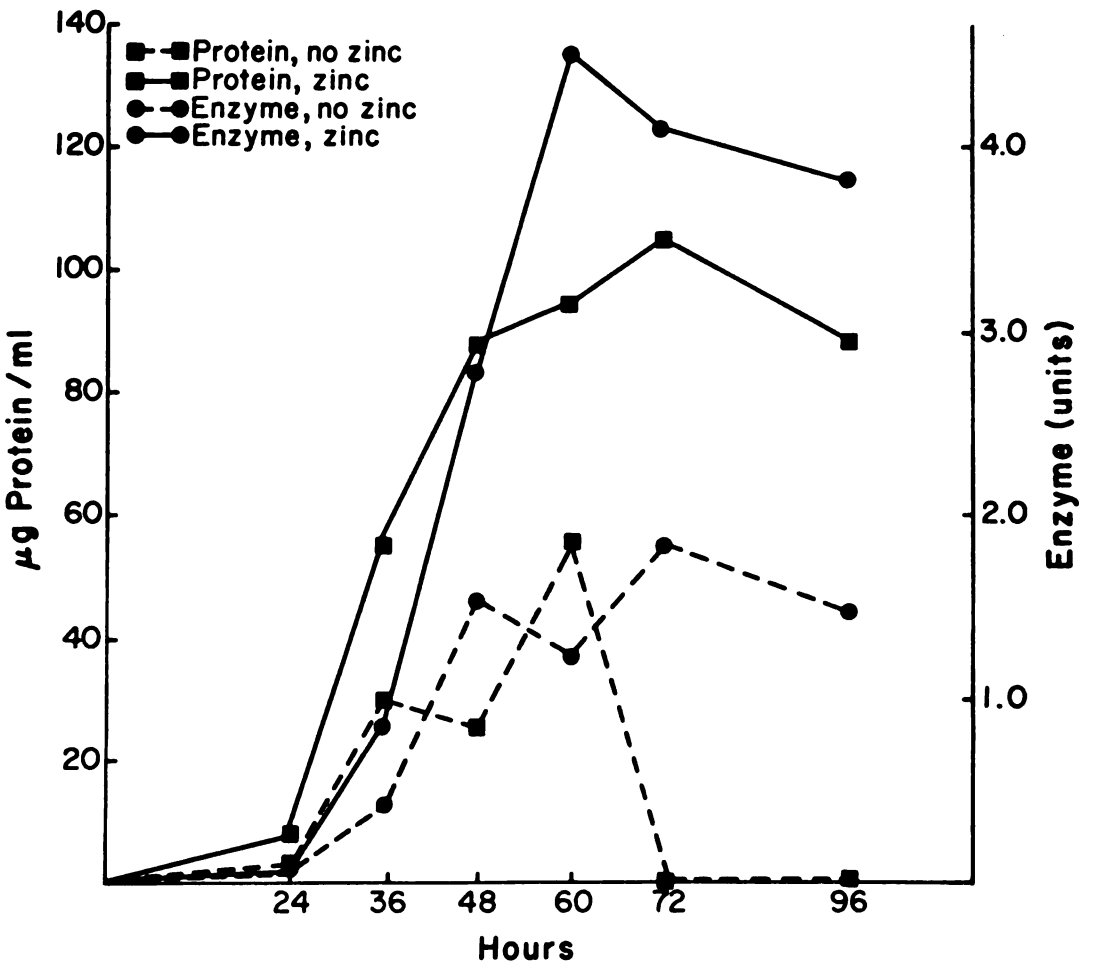


Figure 5.--Growth and enzyme production by Pseudomonas perolens ATCC 10757 in casamino acid medium with and without the addition of 10^{-5} M $ZnCl_2$, (pH 7.5) at 10°C. The medium also contained 4.5 mM $CaCl_2$.

This effect is attributable to the zinc ion and not the chloride ion because with the addition of zinc chloride the chloride concentration increased from 4.500 mM to 4.501 mM. Such an effect by that amount of chloride would have been noticed in medium preparation where a greater amount of calcium chloride than required was added. No such dramatic results were noted. Furthermore, there is precedence for the zinc effect (Conn et al., 1964).

Bacterial Protease Isolation

The procedure established by Buckley (1972) involved ultrafiltration through a UM-2 membrane using the Amicon Ultrafiltration System, Model 402, and then gel filtration via Sephadex G-100, 2.5 x 45 cm column. The results of this isolation procedure are presented in Table 3. It was apparent that the greatest loss of yield was encountered by passage of the supernatant through the UM-2 membrane. At this step, 73.3% of the activity was lost and 63% of the protein that was initially present. It was possible that the membrane may have been deactivating the enzyme, thus the decrease that was encountered. However, a great amount of protein was also retained. The UM-2 membrane had an exclusion limit for molecular weights greater than 2,000 daltons. This membrane would have retained most proteins. Buckley (1972) noted that the somewhat lower yields were obtained as a result of foaming at the membrane. This foaming was attributed to the high nitrogen pressures (80-90 psi) used for concentration. The accumulation of protein at the membrane, forming another layer of the membrane, despite the action

TABLE 3.--Purification data for protease from Pseudomonas perolens ATCC 10757 (from Buckley, 1972).

Treatment	Total Volume (ml)	Total Protein (µg)	Total Activity (units)	Specific Activity (units/µg)	Recovery %	Purification Fold
Crude enzyme	3,880	380,000	27,056	0.0712	100.0	1.0
UM-2 filtrate	3,770	113,100	2,614	0.0231	9.8	0.32
UM-2 concentrate	30	141,000	7,240	0.0513	26.7	0.72
Sephadex G-100	49	2,208	3,270	1.48	12.1	20.8

of a stirrer would also seriously reduce the actual pore size of the membrane. The reduced pore size would lead to foaming and its consequent denaturation.

Batch Adsorption

Because of the change in the enrichment and cultivation techniques that were used in this study, it became apparent that the use of the ultrafiltration step would reduce the amount of enzyme accumulated. The medium of cultivation for the Pseudomonas perolens ATCC 10757 contained protein (casein) hydrolysates as well as the extracellular protease of interest.

DEAE-Sephadex A-50 was equilibrated with 0.1 M Tris-HCl, 0.0045 M $CaCl_2$ at various pH's (5.0-8.0). Prior to addition of the DEAE-Sephadex A-50, the pH of the supernatant was adjusted accordingly. Similarly, the use of increased ionic strength in the eluent was also investigated. Additionally, the effect of pH upon enzyme activity was pursued since it was possible to establish conditions to elute the enzyme but inactivate it through denaturation (Appendix C.1.1).

Figures 6 and 7 illustrate that pH 8.0 and 0.8 M Tris-HCl were required to elute the enzyme DEAE-Sephadex A-50. These conditions were not particularly harsh on the enzyme. In fact, Table 4 demonstrates that while 47% of the protein in the supernatant was lost with this step, the specific activity increased 307%. This increased activity contrasts with the 73% decrease reported by Buckley (1972). A subsequent study demonstrated that 0.8 M NaCl,

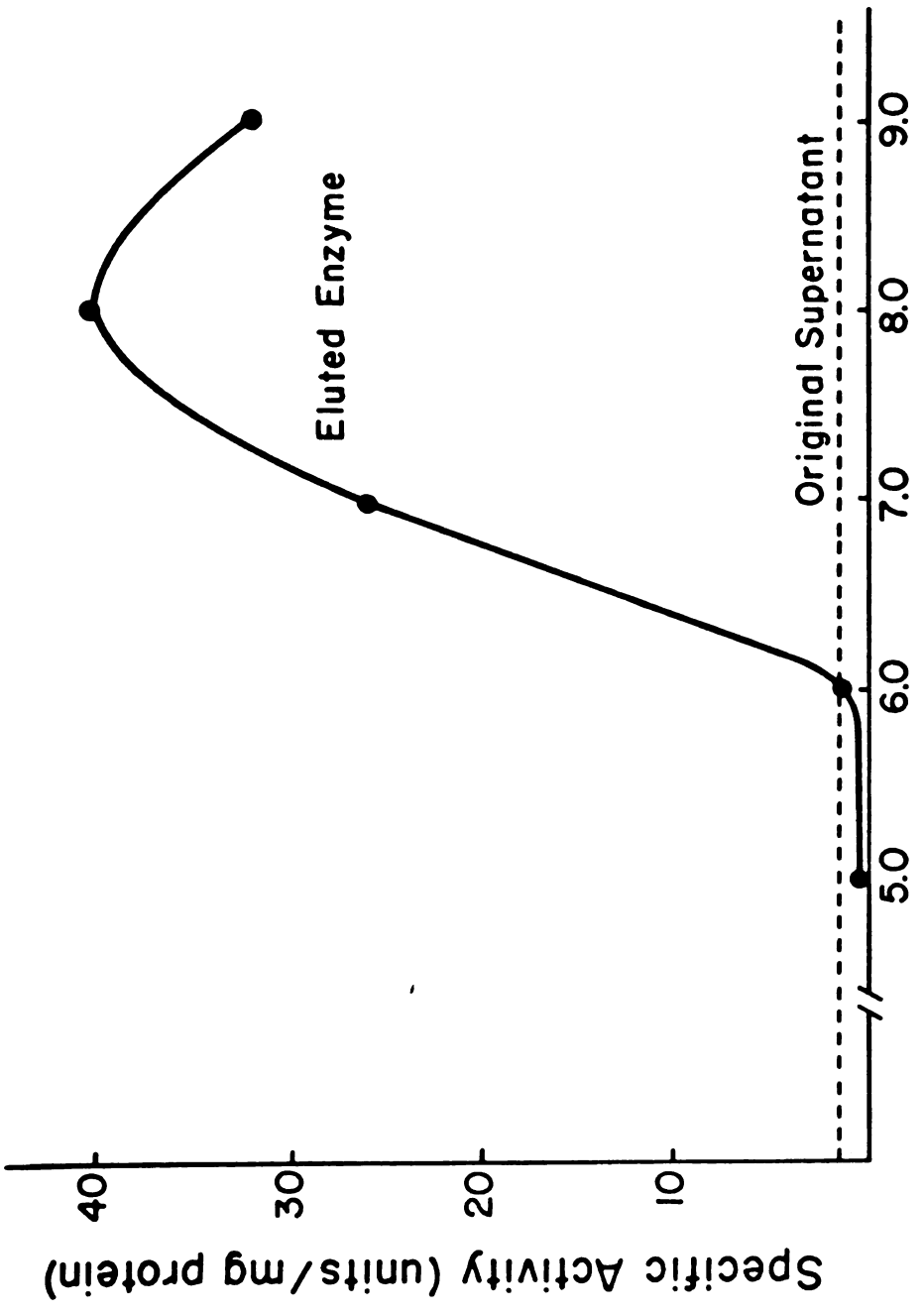


Figure 6.--The effect of pH as an eluent upon enzyme bound to DEAE-Sephadex A-50.

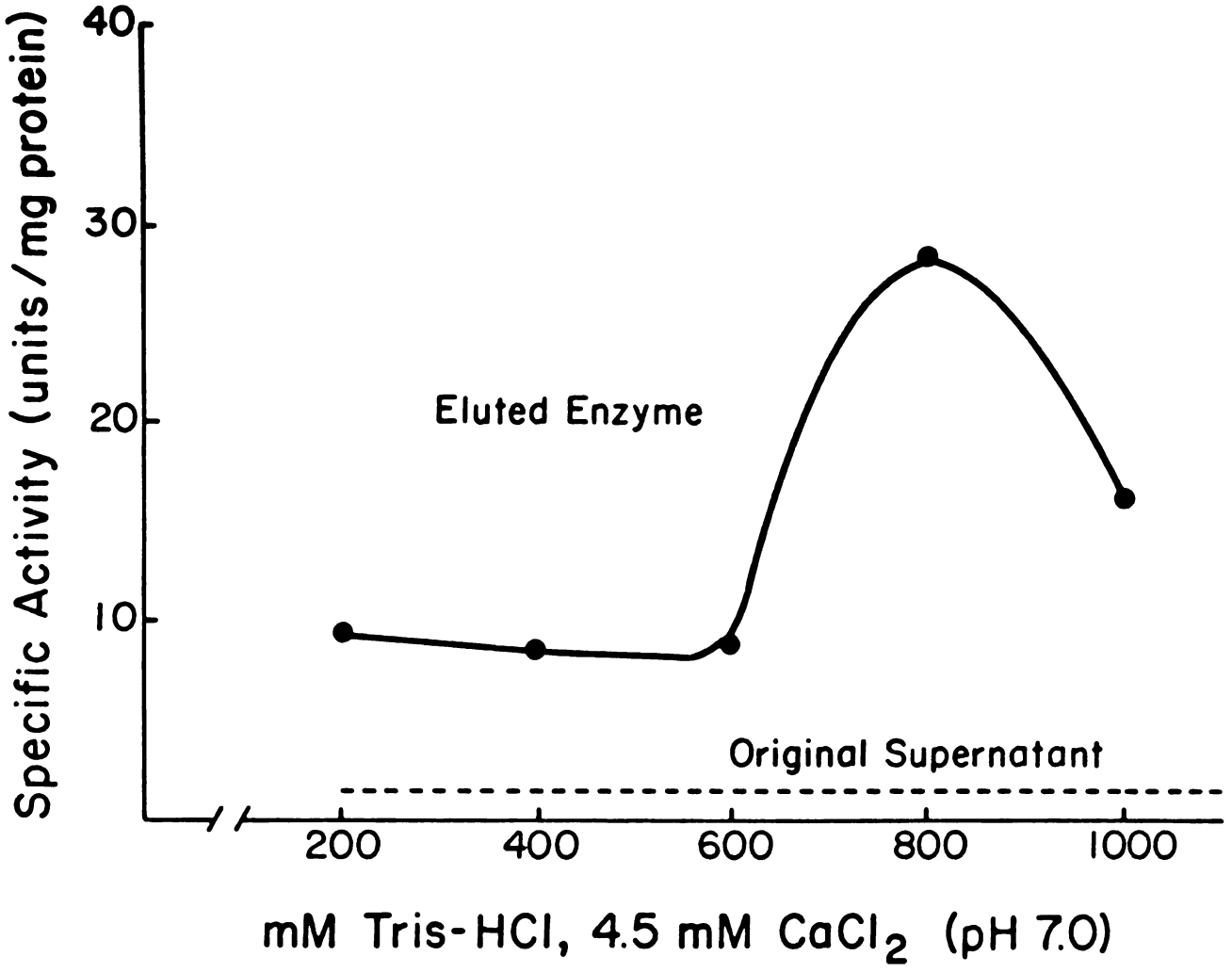


Figure 7.--The effect of eluent ionic strength on enzyme bound to DEAE-Sephadex A-50.

TABLE 4.--Isolation data for the extracellular protease from Pseudomonas perolens ATCC 10757.

Treatment	Total Volume (ml)	Total Protein (mg)	Total Activity (units)	Specific Activity (units/mg)	Recovery %	Purification Fold
Crude enzyme	440	128.4	2,547.6	19.9	100.0	1.0
DEAE-Sephadex	190	21.9	1,339.5	61.1	17.1	3.1
Ammonium sulfate	15	2.3	18,714.0	806.6	2.0	40.5
Sephadex G-100	10	0.01	173.1	1,923.3	0.01	966.5

0.1 M Tris-HCl (pH 8.0) possessed better eluent properties than the above described system (Appendix C.1.2).

The difficulty inherent in altering an enzyme isolation procedure was that the alterations may result in the isolation of another enzyme. This was especially true with the isolation of bacterial enzymes. A number of investigators (Morihara et al., 1963; Arvidson et al., 1972) found a complete spectrum of enzymes present in Pseudomonas aeruginosa and Staphylococcus aureus, respectively. To determine whether the same enzyme had been isolated by the altered procedure, the pH profile of the protease was compared to the reported values of Buckley (1972). The comparison is depicted in Figure 8. While the profiles do not match precisely, Figure 8 does demonstrate that the enzyme was the same. The sharpness of the ion exchange isolated protease may demonstrate that the early work using the ultrafiltration procedure may have had a contaminant protease present. However, other possibilities present themselves such as a protective effect by ions or substrate.

Ammonium Sulfate Precipitation

Standard isolation techniques developed for other enzymes achieved much success through the precipitation of proteins by ammonium sulfate. The DEAE-Sephadex A-50 eluent was treated with ammonium sulfate. Ammonium sulfate was added to 30% saturation, 50% saturation and 70% saturation. The protein content and enzyme activity were monitored for both the pellet and the supernatant. It is apparent from Figure 9 that the 50% ammonium sulfate saturation

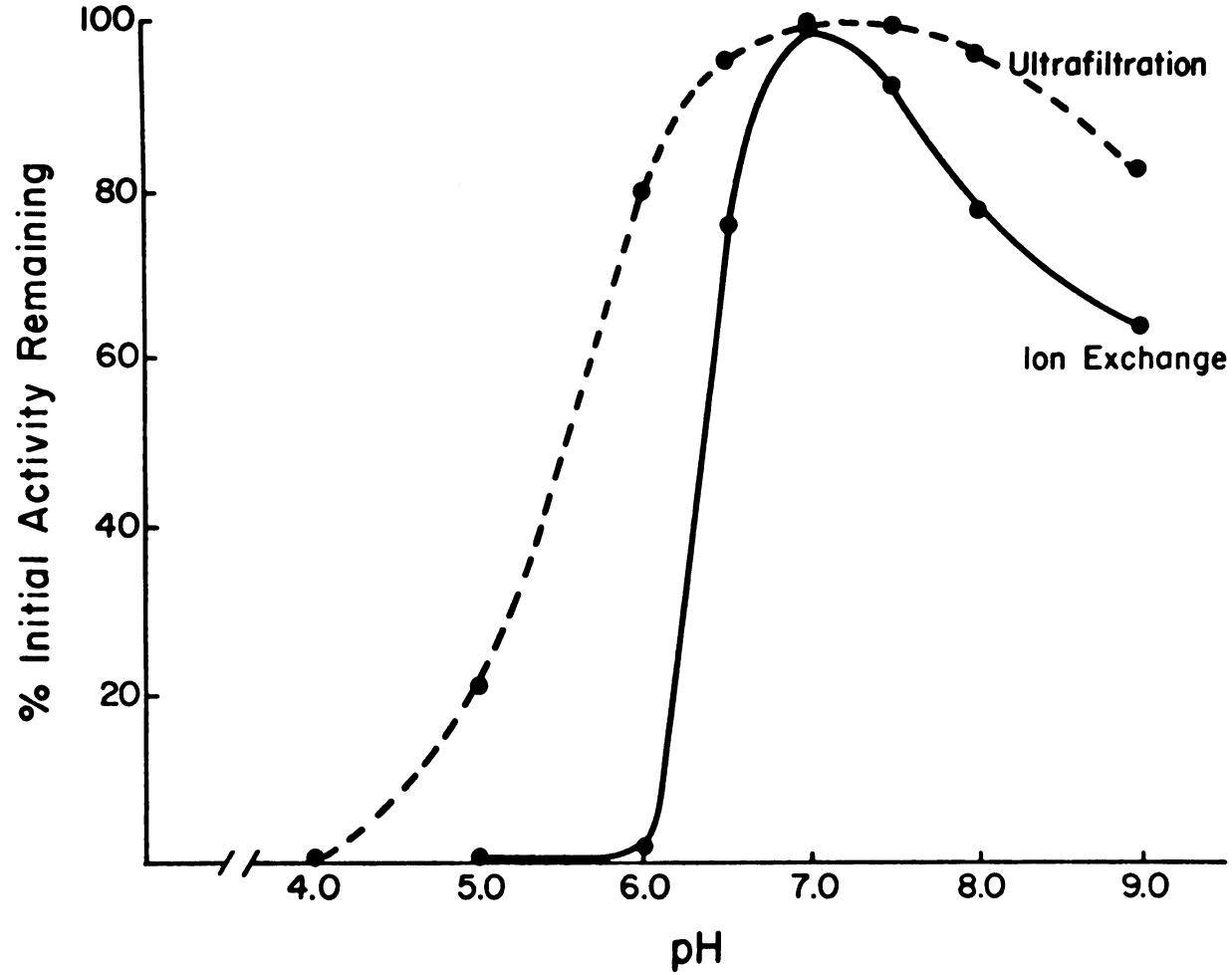


Figure 8.--A comparison of the effect of pH upon the activity of Pseudomonas perolens ATCC 10757 protease isolated by two different methods.

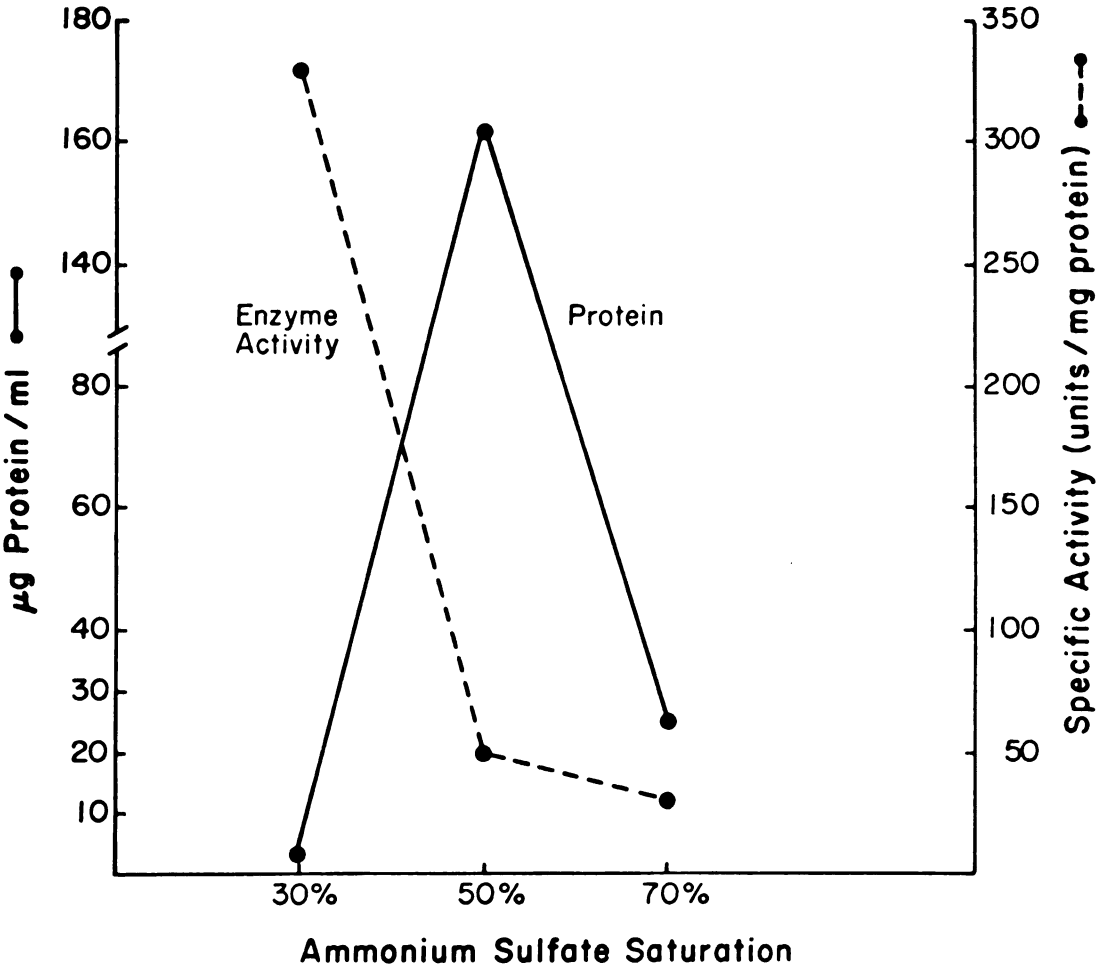


Figure 9.--Ammonium sulfate precipitation of Pseudomonas perolens ATCC 10757 protease from DEAE-Sephadex A-50 eluate. The eluate was 0.8 M NaCl, 0.1 M Tris-HCl (pH 8.0) and 0.0045 M $CaCl_2$.

cut had the highest specific activity. The inclusion of this step into the enzyme isolation protocol resulted in a severe reduction in the amount of protein recovered. However, the specific activity increased 400 fold over the initial value. These results are presented in Table 4. (See also Appendix C.2.)

Molecular Exclusion Chromatography

On a 2.5 x 45 cm column of G-100 Sephadex the enzyme fractions were eluted at tubes 48-52 by 0.1 M Tris-HCl, 0.0045 M $CaCl_2$ (pH 7.5). The tubes consisted of 10 ml of eluent. This is very similar to the results reported by Buckley (1972) Figure 10 presents the results of Sephadex G-100 exclusion chromatography. A protein of high specific activity was isolated along with the disclosure of a second activity peak. The presence of the second peak was to be expected for a bacterial preparation of this type. This second peak was not pursued since its activity was less than the peak of initial interest.

The activity peaks were examined via sodium dodecyl sulfate polyacrylamide gel electrophoresis. These results will be reported later in this study.

Enzyme Parameters

The pH optimum for the enzyme was between pH 7.0 and 8.0, which agrees with the results of Buckley (1972). The enzyme was not inhibited by cysteine, iodoacetate, phenyl methyl sulfonyl fluoride, dithiothreitol or p-chloromercuribenzoate, as previously reported. The above compounds were not inhibitory at the 5.0 mM level. The

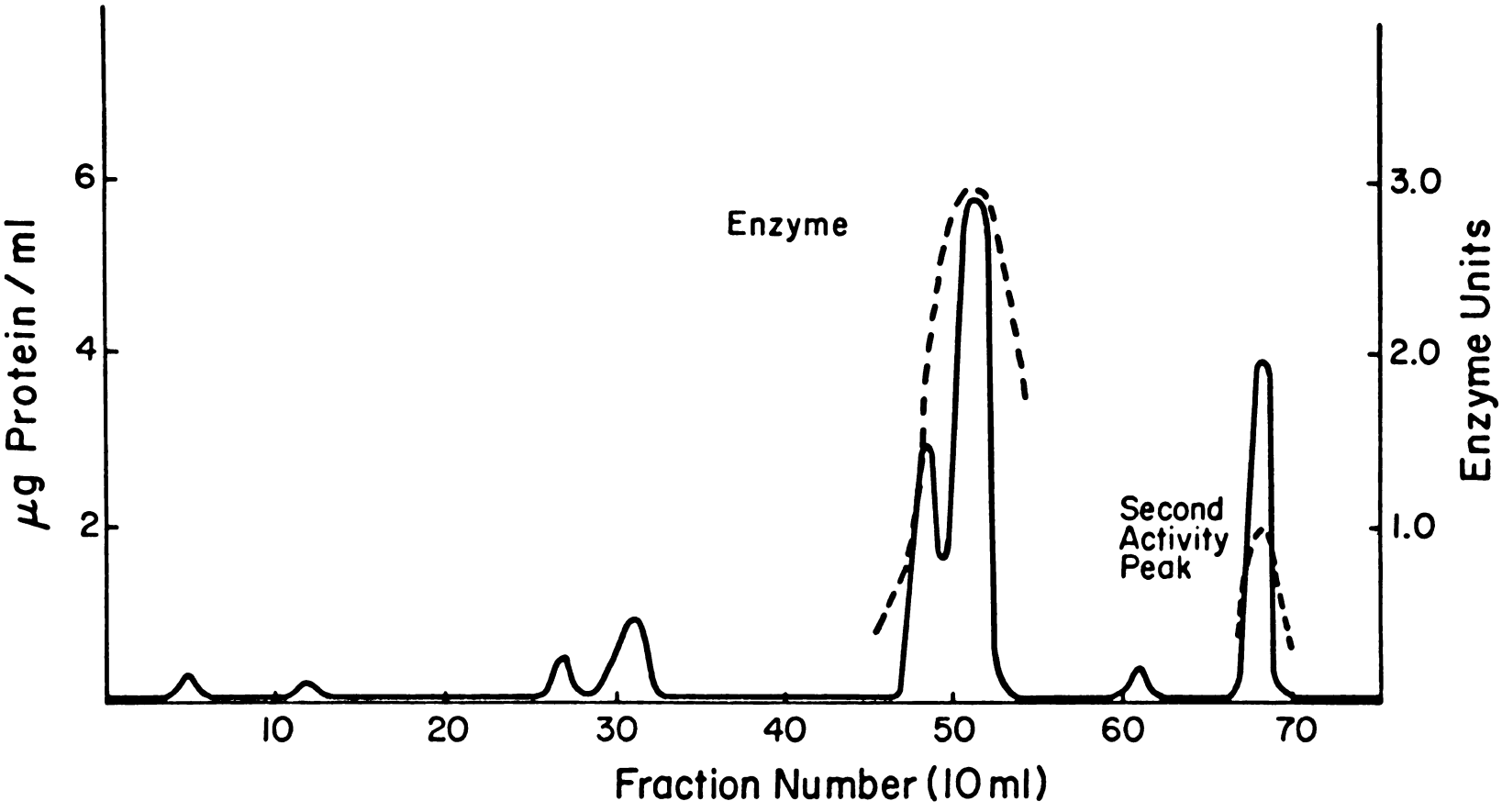


Figure 10.--Sephadex G-100 separation of Pseudomonas perolens ATCC 10757 extracellular protease. Conditions defined in text. Protein determination (———) and enzyme activity (- - -).

temperature maximum was not tested but was found by Buckley (1972) to be 35°C.

EDTA Inhibition

EDTA inhibition was demonstrated by Buckley (1972) for Pseudomonas perolens and for other pseudomonads by Morihara et al. (1963), Muriyama et al. (1969) and Porzio and Pearson (1975). Buckley L972) observed 50% inhibition upon addition of 0.1 mM EDTA. As a further indication of the identity of the protease isolated by the new enzyme protocol, its inhibition by EDTA was determined. The results of this determination are presented in Table 5. It seems that the addition of EDTA does inhibit proteolytic activity. Fifty percent inhibition occurred between 5 and 10 mmoles EDTA. This meant that the enzyme isolated via the new protocol was at least 50 times more sensitive to EDTA than previously described (Buckley, 1972).

TABLE 5.--The effect of EDTA addition upon the activity of extracellular protease isolated from Pseudomonas perolens ATCC 10757.

EDTA mmoles	Enzyme Activity		Inhibition %
	Units	Specific Activity	
0	107.7	651.0	--
1	102.9	621.6	4.5
5	71.0	428.9	34.1
10	5.9	35.7	94.5
20	2.2	13.1	98.1

This occurrence may have been the result of the removal of all traces of Ca^{++} during the test to avoid affecting the results. Similarly, if this enzyme was of greater purity than the previous isolation, it should demonstrate greater sensitivity.

The fact that as the millimolar concentration of EDTA increased, 100% inhibition was approached asymptotically, may demonstrate that the Ca^{++} is tightly bound to the enzyme and is not capable of being affected by EDTA. Possibly total inhibition would occur only with denaturation or complete unfolding of the enzyme (Appendix C.3.1).

The ability of $CaCl_2$ to reactivate the enzyme is depicted in Figure 11. Though not shown in Figure 11, the enzyme was never fully reactivated beyond the 50 mmoles of Ca^{++} level. This again may demonstrate that the Ca^{++} is required for stability and not for activation. If this is true, then the Ca^{++} must be incorporated into the enzyme at the time of synthesis and not afterwards as in this test (Appendix C.3.2).

Enzyme Kinetics

In a series of papers (Morihara et al., 1969; Morihara and Tsuzuki, 1971; Morihara, 1974) the specificity of bacterial protease activity upon synthetic substrates was discussed. The use of a synthetic substrate would allow the kinetics of the protease to be assessed. The casein substrate normally used does not lend itself to kinetic work because the molecular weight is not known specifically.

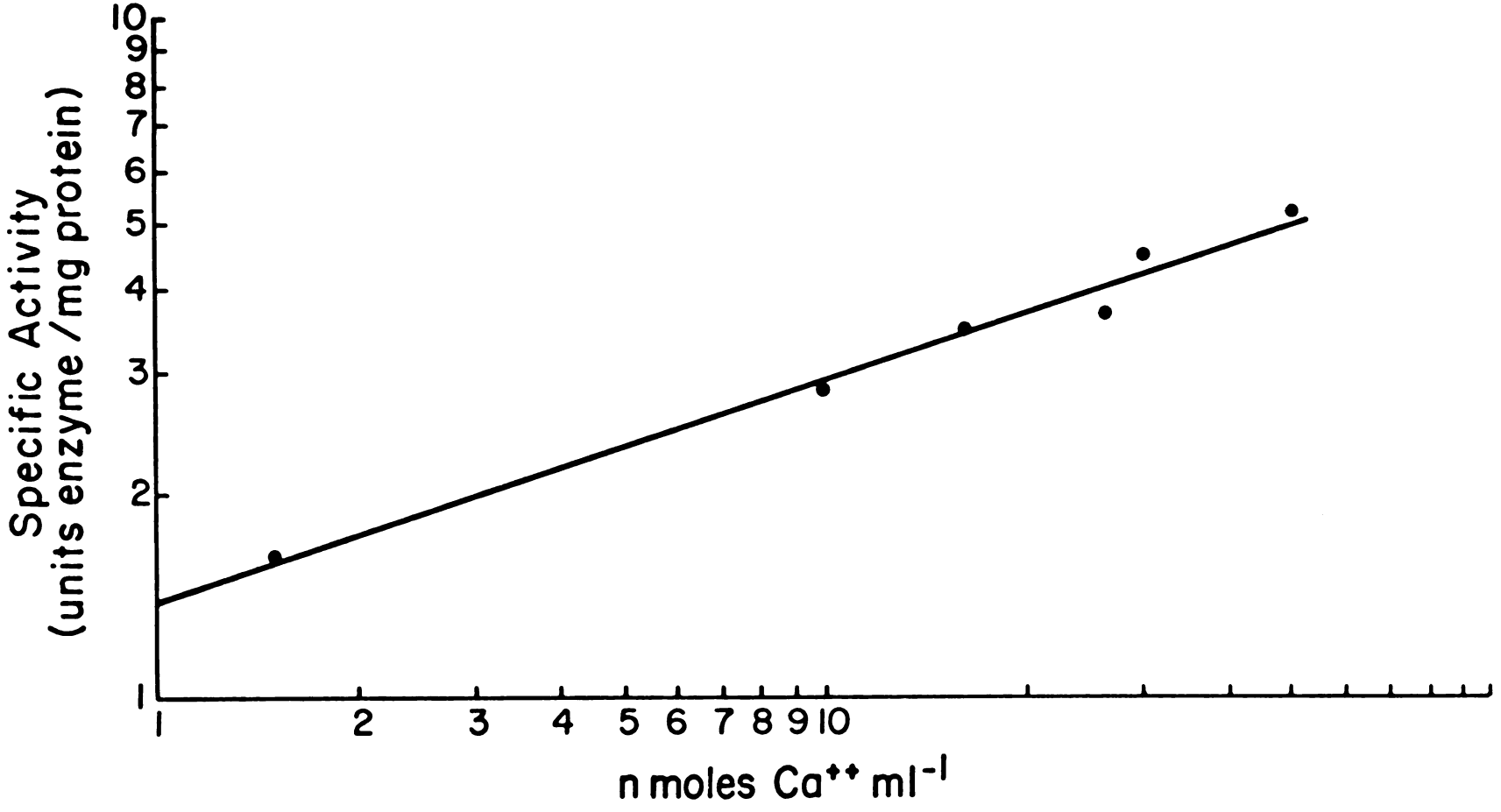


Figure 11.--The effect of Ca^{++} addition upon enzyme activity of Pseudomonas perolens ATCC 10757 protease inhibited by 25 mmoles EDTA.

Two synthetic substrates were used to assess protease activity, N-carbobenzoxy-L-alanyl-L-leucine and N-carbobenzoxy-glycyl-L-leucine. The split of the peptide bond was monitored by the ninhydrin method.

Early in this study it was determined that N-carbobenzoxy-glycyl-L-leucine was the better substrate, therefore it was used primarily in this study. The fact that this substrate was attacked by the protease supports the results reported by Buckley (1972) concerning collagen digestion. He demonstrated that hydroxyproline was released by proteolysis of collagen, however, there was no free hydroxyproline. He postulated that hydroxyproline was released in the form of peptides.

Glycine represents nearly one-third of the total amino acid residues (32.7%) in bovine intramuscular and rat skin collagen. However, bovine intramuscular and rat skin collagen contain only 2.5% leucine (Bodwell and McClain, 1971). This means that the molecular chain sequence of collagen approximates the following (Forrest et al., 1975):

- X - Gly - Pro - HydroxyPro - Gly - X - .

Thus, in the above sequence one-third of the remaining residues are other than the normal residues, or out of 1,000 residues only 25 out of 333 can be leucine. Since the substrate N-CBZ-glycyl-L-leucine was attacked the bond would be cleaved but the likelihood of free hydroxyproline in the supernatant was very small. Therefore, the hydroxyproline that Buckley (1972) monitored would be in the peptide form.

Figure 12 presents a Lineweaver-Burke plot of the hydrolysis of N-CBZ-glycyl-L-leucine. The K_m and V_{max} for this substrate were 2.6 mM and 169.5 μM leucine ml^{-1} $minute^{-1}$, respectively. According to Morihara (1974) this is a factor of ten lower than the K_m exhibited by Bacillus subtilis neutral protease on the same substrate. Morihara (1974), in characterizing the neutral proteases, has suggested that these enzymes are specific against hydrophobic or bulky amino acid residues, such as leucine or phenylalanine at the alpha amino side of the splitting point (Appendix C.3.3).

Sodium Dodecyl Sulfate Polyacrylamide Gel Electrophoresis

The SDS gel depicting the final product of the isolation procedure is shown in Figure 23. The molecular weight of the protease was between 35,000 and 40,000 daltons. This molecular weight is within the range described by Morihara (1974) as typical for neutral proteases.

Unit Definitions

Enzyme Activity

One unit is expressed as the number of μg of tyrosine equivalents (Lowry et al., 1951) released per milliliter of enzyme solution per minute at 35°C using 2% (w/v) casein solution.

Specific Activity

One unit is expressed as the number of units of enzyme activity per milligram of protein.

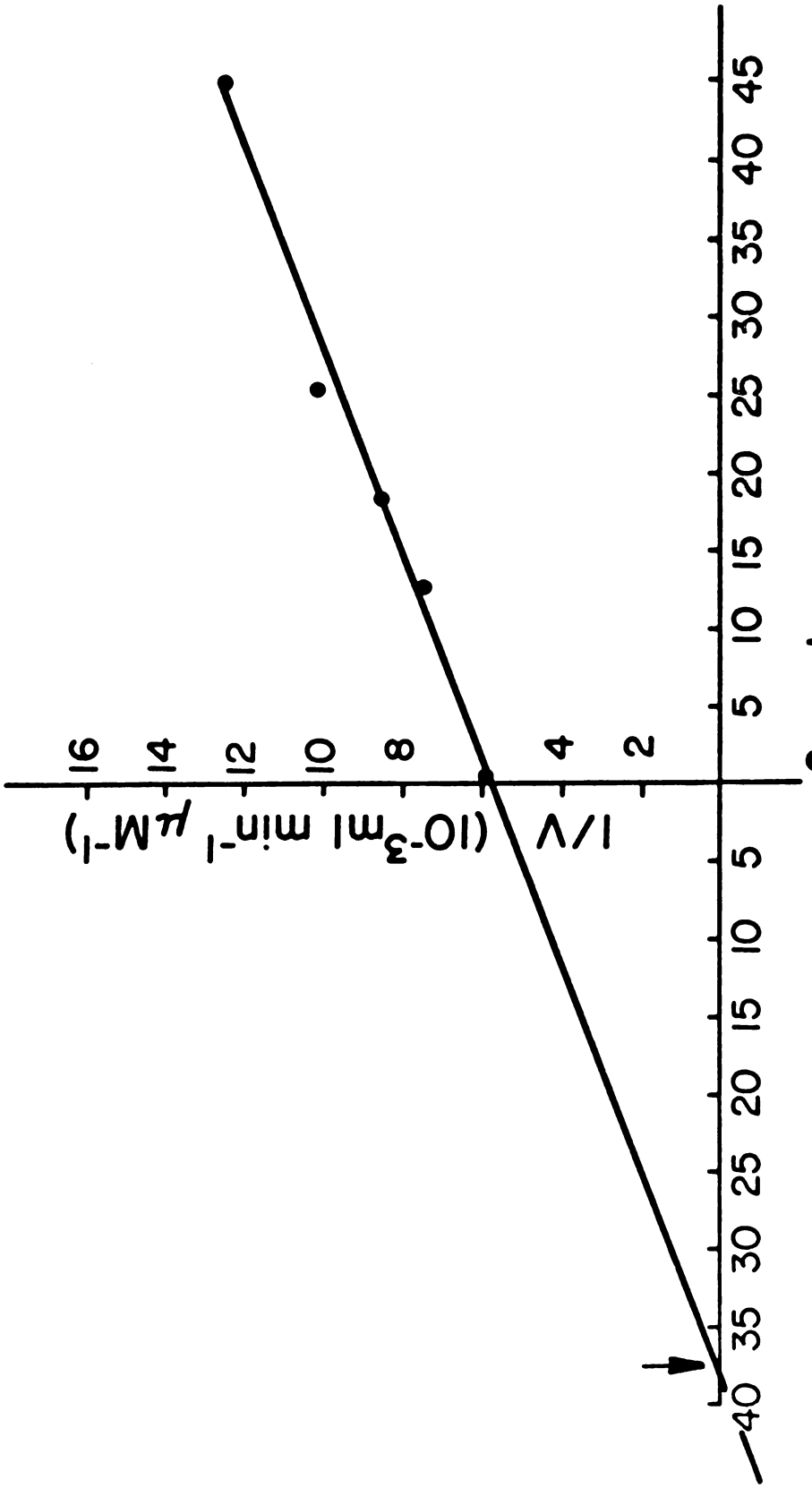


Figure 12.--Lineweaver-Burke plot of N-CBZ-glycyl-L-leucine hydrolysis by Pseudomonas perolens ATCC 10757 protease.

Vertebrate Skeletal Muscle Proteases

Calcium Activated Sarcoplasmic Factor and Fasting

Twelve rabbits were selected for the Calcium Activated Sarcoplasmic Factor (CASF) study. All of the rabbits weighed approximately three kilograms and were adult males. The twelve rabbits that were selected for the CASF study demonstrated similar weight gains during a two-week preliminary assessment period. From these twelve New Zealand White rabbits eight were selected at random for the fasting study and the remainder were fed a normal regimen of rabbit chow and water. The fasted rabbits received only water for 28 days. The animals were weighed regularly and blood samples were taken after the weighing.

Physiological Effects of Fasting

At the beginning of the CASF study the average weight of the rabbits (n = 12) was 3,187.9 g (± 123.6 g). The average daily weight gain was 7.6 g (± 8.2 g). The standard deviations in weight and average daily weight gain for the study group were half of the values for the entire population of rabbits (n = 26). This would demonstrate that the population of rabbits chosen for the CASF study was more homogeneous than the rabbits of the KAF study.

Figures 13 and 14 dramatically illustrate the effect of this 28-day study on live animal weight and average daily weight gain, respectively. At the completion of the study the fasted rabbits had an average weight of 2,095.9 g (± 236.5 g), whereas the fed rabbits weighed 3,475.3 g (± 34.4 g). These weights resulted

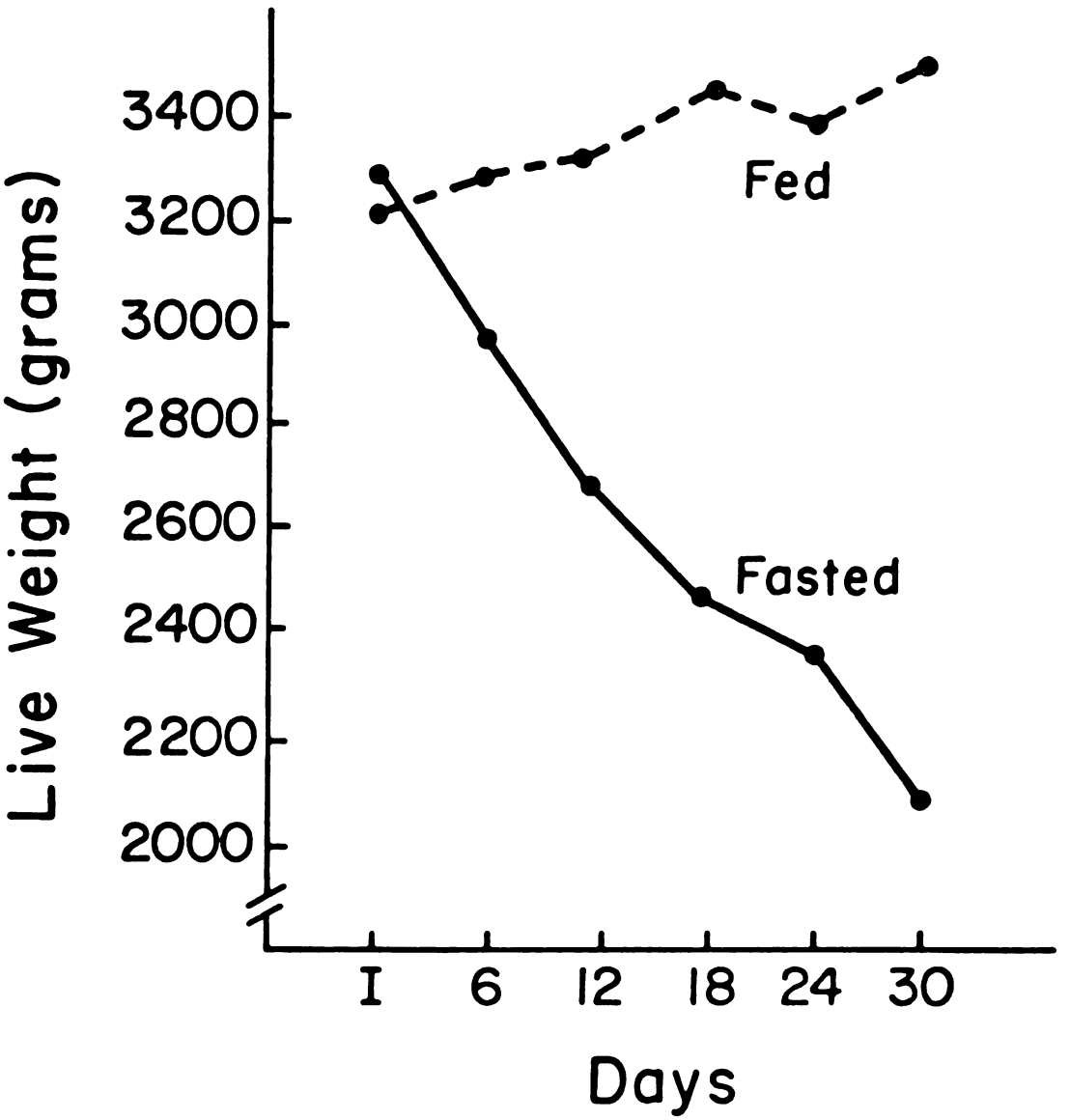


Figure 13.--The effect of extended fasting upon the live weight of adult male New Zealand rabbits.

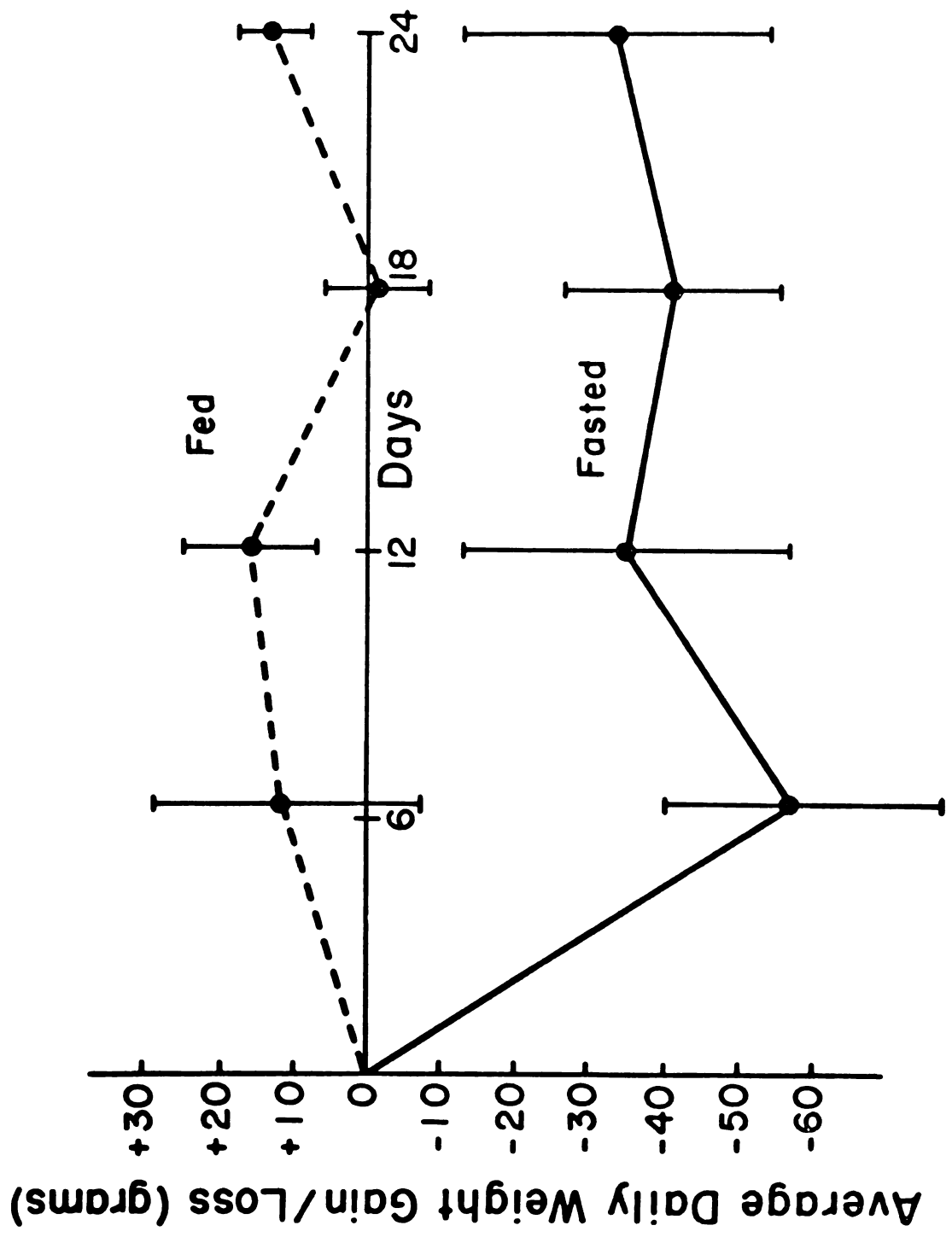


Figure 14.--The effect of extended fasting upon the average daily weight gain of adult male New Zealand rabbits.

from an average daily weight loss of 42.6 g (± 5.0 g) for the fasted rabbits and an average daily weight gain of 9.8 g (± 9.8 g) for the ad libitum fed rabbits. The results from the fed rabbits indicated that the rabbits selected for this study had reached or were approaching the plateau of their growth curve (Forrest et al., 1975).

The effect of extended fasting was monitored on individual muscles (semitendinosus and longissiumus), two organs (heart and liver), as well as live animal weight. These data are presented in Table 6. The difference in the muscle weights was significant at $p \leq 0.01$; however, for the heart the significance level was $p \leq 0.05$. The liver weight difference was significant at $p \leq 0.01$. The fasted rabbit muscles were 43% and 54% lower in weight than the fed rabbit muscles for the semitendinosus and longissiumus muscles, respectively. This corresponded with the 40% difference between the fasted and fed rabbits in final live weight. The heart muscle exhibited the least difference between the fasted and fed rabbits, 29%. The liver demonstrated the greatest effect from fasting with a 64% difference between the fasted and fed animals. Millward (1970a, 1970b) reported different results. After three days of fasting he found a slight increase in catabolism in the liver but a 75% increase in myofibrillar tissue catabolism. The liver proteins have a steady state equilibrium (K_s/K_d) of 0.46 compared to 0.56 for the myofibrillar proteins (i.e., there was a greater turnover of liver protein than myofibrillar protein in the normal state).

TABLE 6.--The effect of fasting upon the semitendinosus and longissimus muscles, the heart and the liver.

Treatment	Muscle Weight (g)		Organ Weight (g)	
	Semitendinosus	Longissimus	Heart	Liver
Fasted	10.6 (± 1.4)	47.2 (± 11.4)	4.7 (± 0.7)	27.8 (± 4.4)
Fed	18.5 (± 2.7)	103.4 (± 5.5)	6.6 (± 0.4)	77.1 (± 6.4)

Biochemical Effects of Fasting:
Blood Parameters

In order to assess the effect of fasting upon the whole animal three blood parameters were assayed. The glucose level of the blood was monitored to observe if gluconeogenesis occurred. The free amino acids (total) were assayed to determine if amino acid mobilization had occurred to provide the carbon skeletons for gluconeogenesis (Young, 1970). And the nonesterified free fatty acids were monitored to maintain a check on the other sources of energy in the animal body.

Glucose.--The glucose level of blood serum was assayed by the glucose oxidase technique. The results of the assay are presented in Figure 15. The bleeding times were approximately six days apart. Because of the large variation that was encountered for the data points, the difference between the two treatments was nonsignificant (Appendix D.2.1). However, while the glucose levels that were observed in the fasted animals did approximate the normal fed state, the normal state stability was absent. This fluctuation was determined to be an actual fact by the nonesterified free fatty acid assay since a drop in glucose level in the blood should act as a signal for increased free fatty acids. The glucose level was maintained by increased gluconeogenesis in the liver and kidney. The source of precursors is adipose tissue which furnishes glycerol (Newsholme and Start, 1973) and muscle which furnishes amino acids and lactate (Newsholme and Start, 1973; Bartley et al., 1968).

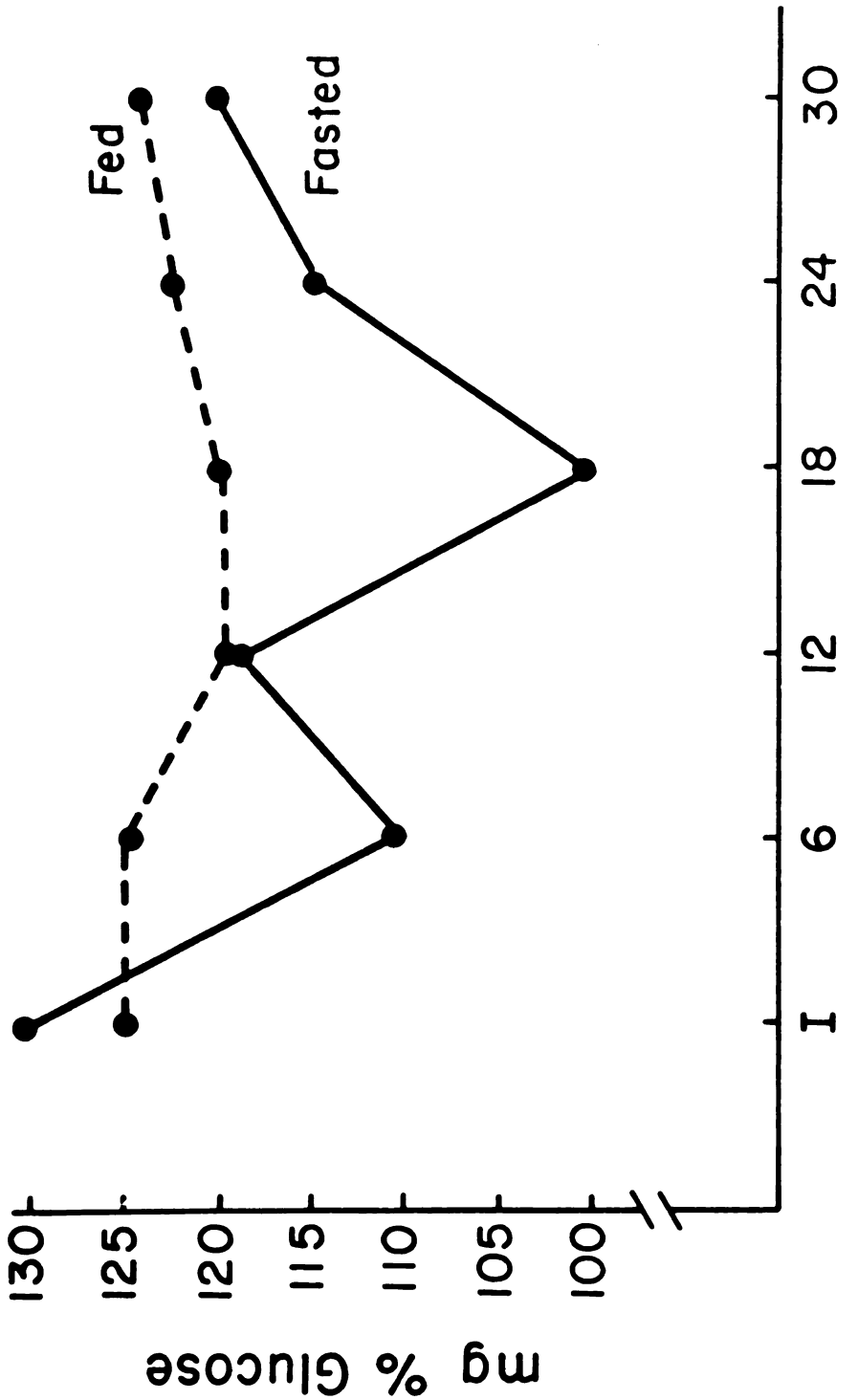


Figure 15.--The blood serum glucose levels of fasted and fed adult male New Zealand rabbits.

Total free amino acids.--It is apparent from Figure 16 that the total free amino acid level in the blood of both fed and fasted rabbits was the same. As mentioned earlier, the amino acids were mobilized primarily from skeletal muscle to the liver and kidney for gluconeogenesis. The glucose synthesized serves as an intermediate energy source. This study assayed total free amino acids. Felig and Wahren (1974) reported that alanine and glutamine were the major amino acids released during fasting. During a 3.5 day fast, Block and Hubbard (1962) found that alanine and glutamine decreased by 20 and 53%, respectively. The same investigators also reported an increase in leucine, isoleucine and valine. These branched chain amino acids indicate muscle protein catabolism (Munro, 1970).

Nonesterified free fatty acids.--The amount of free fatty acids reaching the liver and other organs should increase as the glucose level in the blood decreases. The glycerol in the liver and kidney is a gluconeogenic precursor and the free fatty acids are metabolized in various tissues to spare glucose for the brain. (Although the brain will use ketones as a substrate, since the drain on protein reserves would be tremendous to furnish precursors for gluconeogenesis, according to Newsholme and Start, 1973.) The results of the nonesterified free fatty acid assay in the fasted and fed rabbits are presented in Figure 17. The supposition posited earlier concerning the interrelationship between blood serum glucose and free fatty acids was substantiated by the data depicted in Figure 17. In spite of a large vairance in the values, the free fatty acids did increase in the blood when glucose decreased.

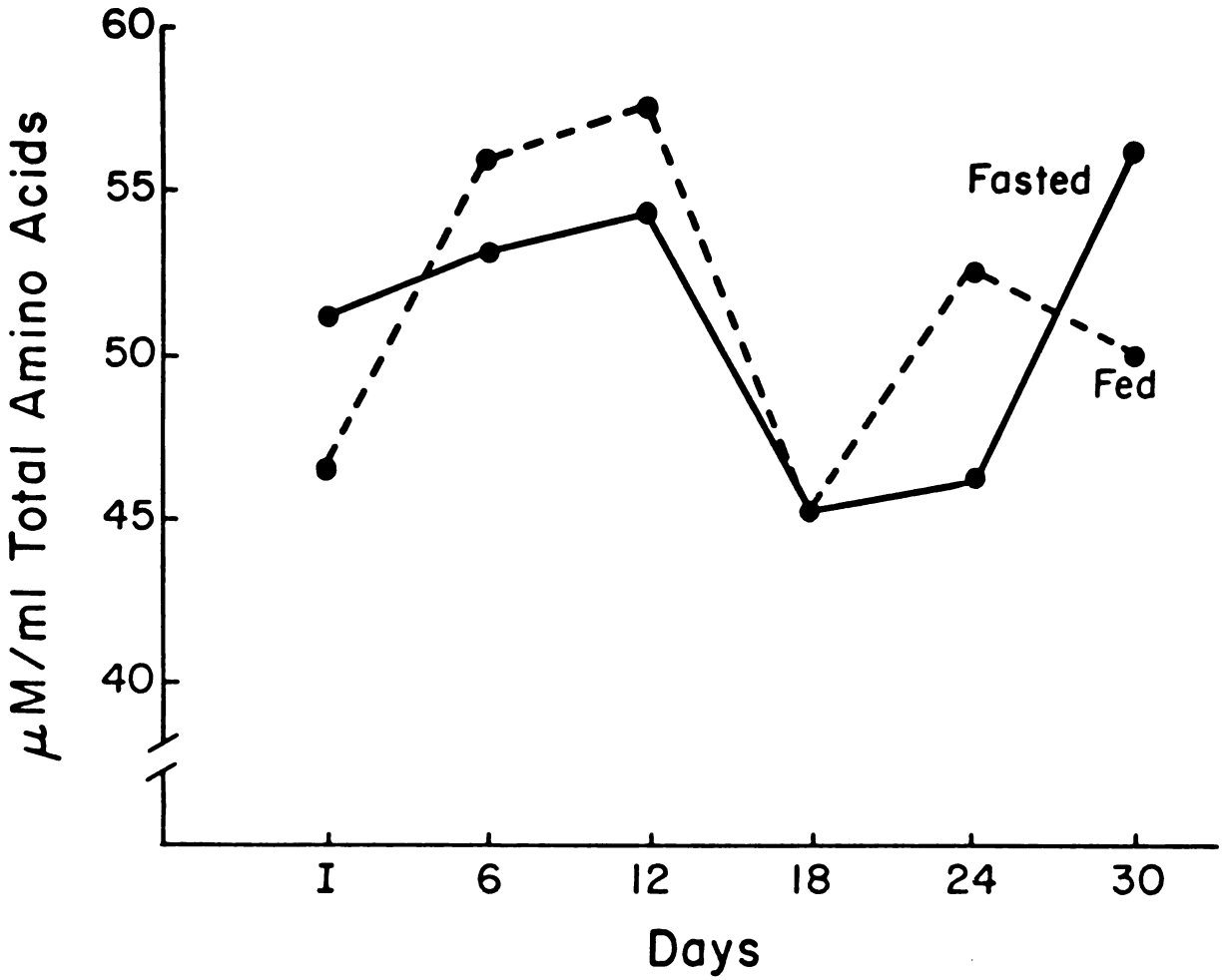


Figure 16.--The total free amino acid levels of blood serum from fasted and fed adult male New Zealand rabbits.

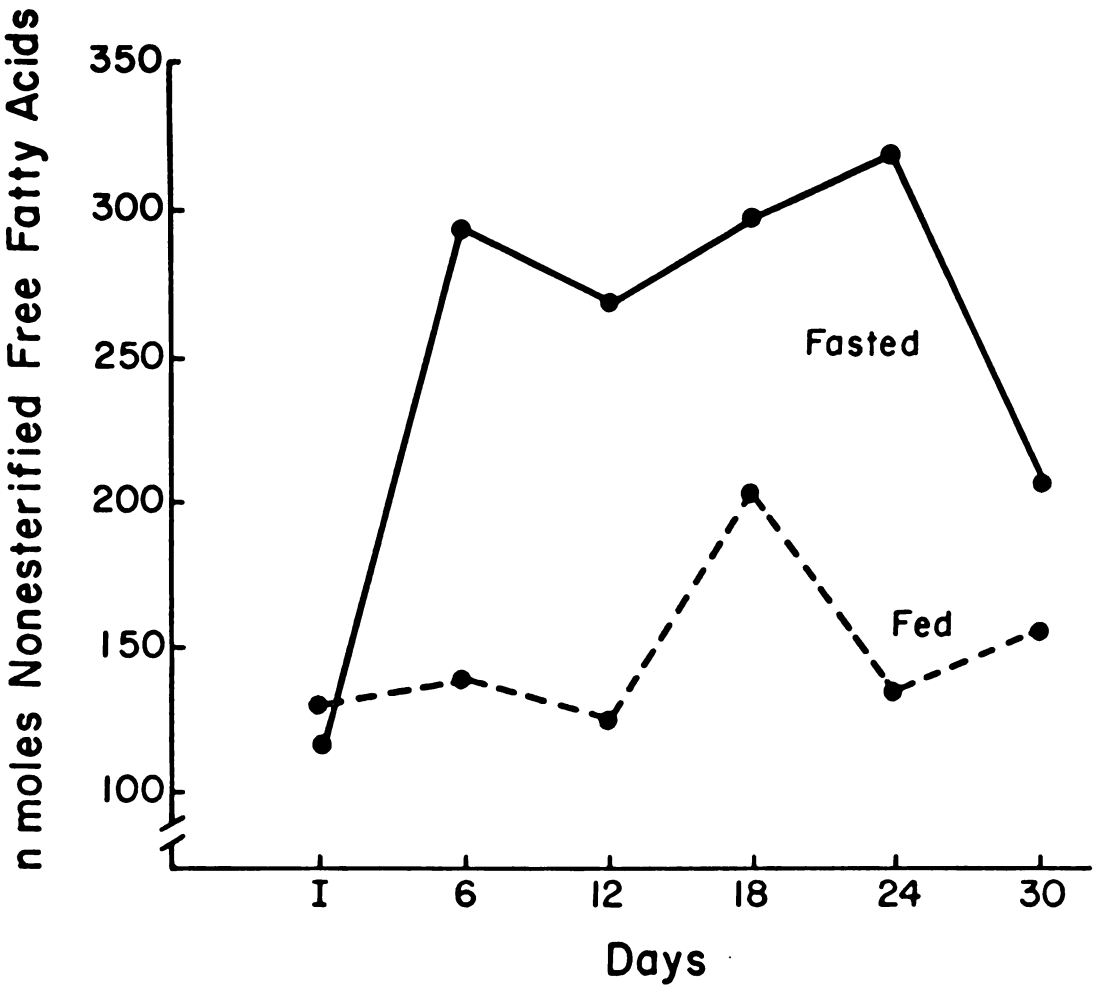


Figure 17.--The nonesterified free fatty acid levels in the blood serum of fasted and fed adult male New Zealand rabbits.

Muscle Parameters

Since skeletal muscle appears to be the major reservoir or pool for amino acids, the protein content of two muscles, the semitendinosus and longissimus, was determined. The effect of fasting upon the major protein components of muscle was observed also. The results for the above mentioned muscles are depicted in Figures 18 and 19. Because the data are presented in a milligram of protein per gram of tissue basis there appear to be no significant differences between protein component fractions in muscles from animals in the two treatment groups. The total protein content as depicted in the figures does not demonstrate the dramatic change that has occurred as observed in the live weights and muscle weights of the animals. However, Table 7, which expresses the results on a per muscle basis, shows the effects of fasting as noted in other parameters. It is

TABLE 7.--The effect of fasting upon the total, myofibrillar, sarcoplasmic stroma proteins and non-protein nitrogen levels in the semitendinosus and longissimus muscles of adult male rabbits.

Muscle	Type	Protein (g/muscle)				
		Total	Myofibrillar	Sarcoplasmic	Stroma	NPN
Longissimus	Fed	25.0	11.6	5.5	3.7	4.0
	Fasted	10.6	4.5	2.6	1.9	2.0
Semitendinosus	Fed	4.3	2.1	1.0	0.5	0.6
	Fasted	2.3	1.1	0.5	0.4	0.4

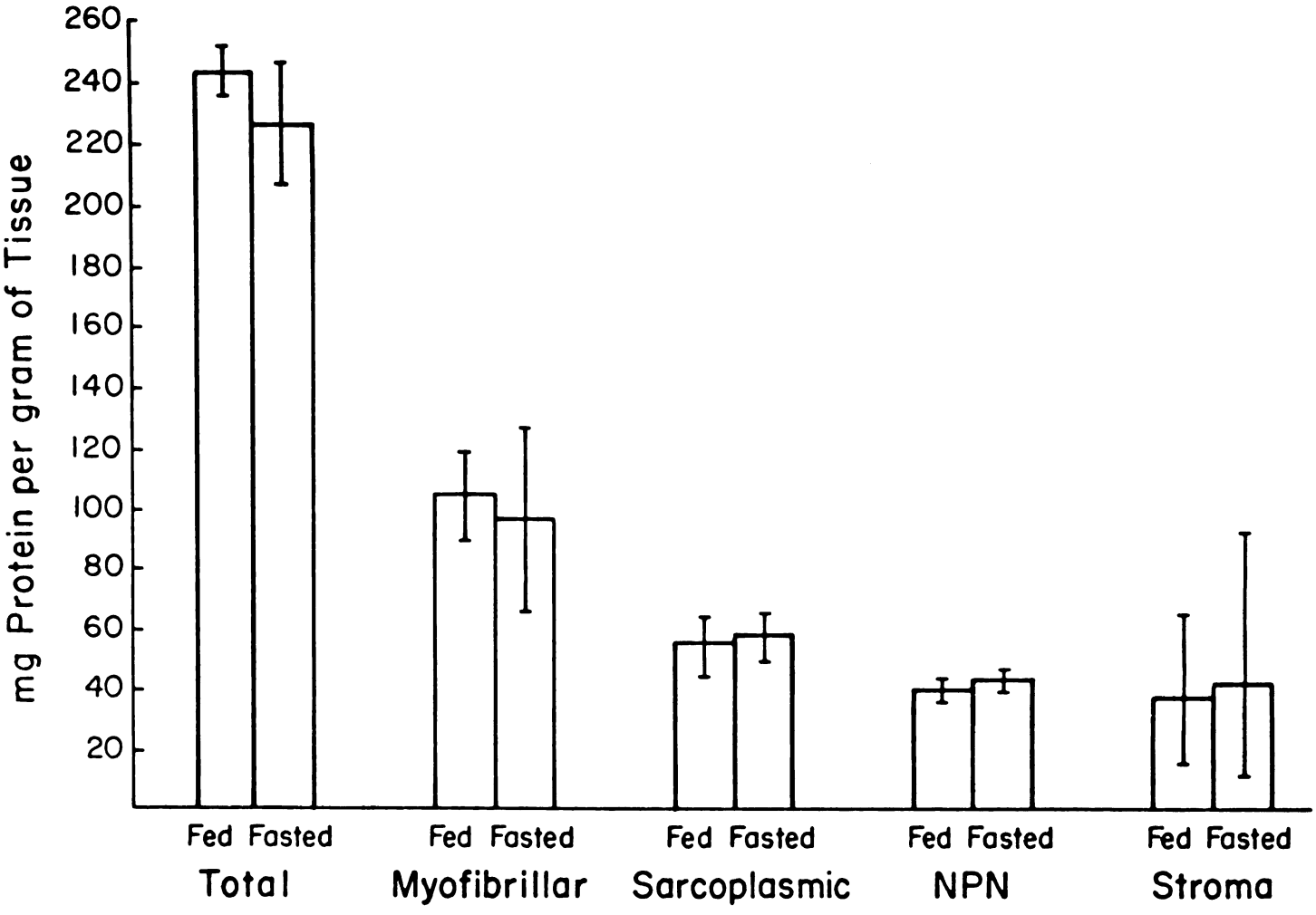


Figure 18.--The effect of fasting upon the total protein content and individual protein components of the longissimus muscle from adult male New Zealand rabbits.

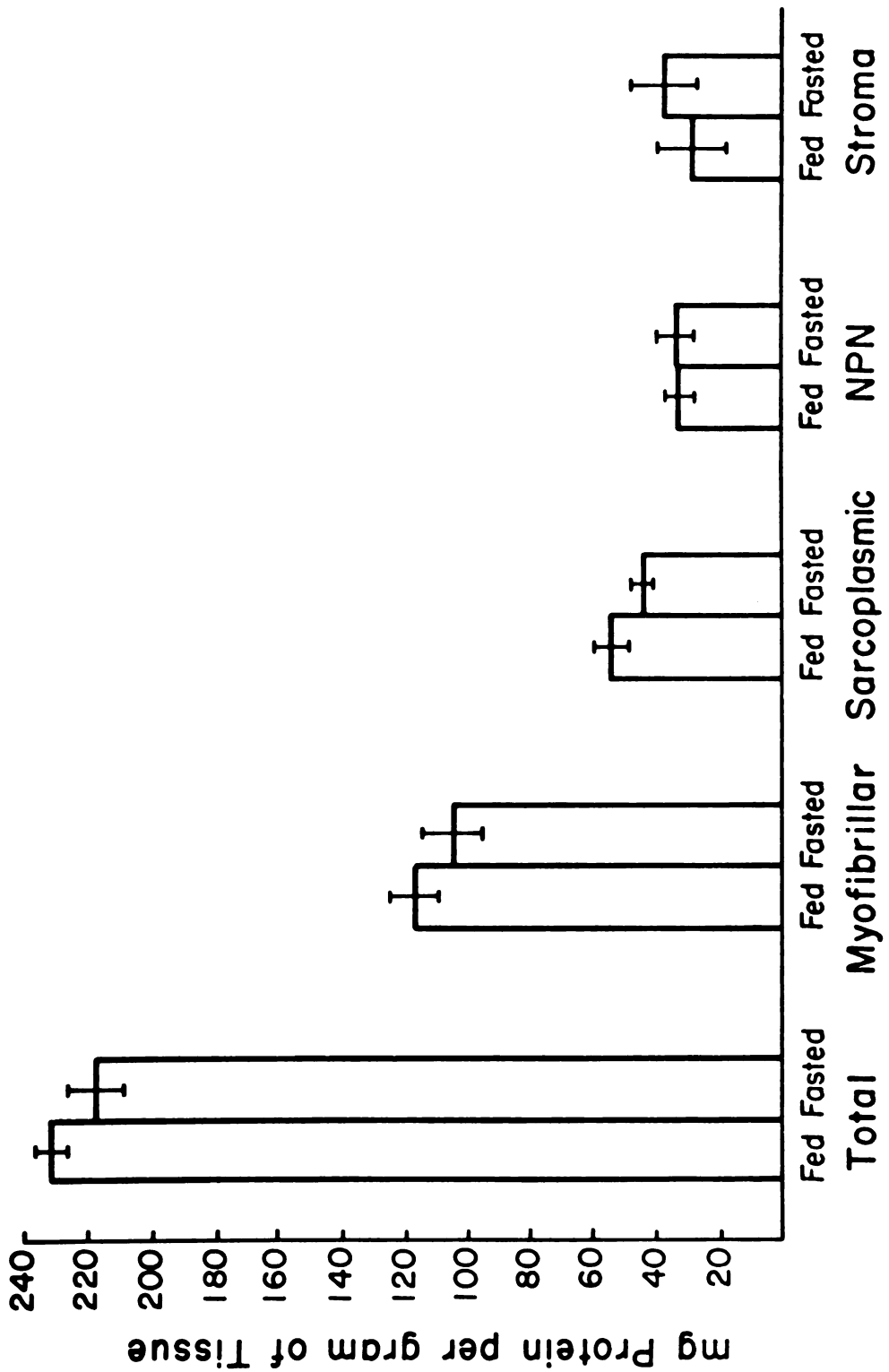


Figure 19.--The effect of fasting upon the total protein content and individual protein components of the semitendinosus muscle from adult male New Zealand rabbits.

apparent from these data that all of the component fractions were affected by fasting. The total protein in the longissimus and semitendinosus decreased 58 and 54%, respectively. The longissimus muscle showed increases in the sarcoplasmic and stromal proteins and non-protein nitrogen with the myofibrillar protein fraction decreasing. This is depicted in Figure 18. The semitendinosus muscle showed increases in the NPN and stromal fractions.

The difference in observed response between the two muscles may be attributed to two possibilities. The removal of the longissimus in its entirety was difficult to accomplish and some variation was to be expected, 11.4 and 5.5 grams, respectively, in fasted and fed rabbits. However, the difference in weights between these two muscles was still significant. The second possibility involves an anthropomorphic interpretation. The longissimus is primarily a structural or postural muscle in function, whereas the semitendinosus is an essential leg muscle. It would be to the animal's disadvantage, individual and evolutionarily, to limit degradation or catabolism in a "fright or flight" muscle and not in a postural muscle. However, both of these explanations are conjectural.

Isolation of Tissue Proteinases

Calcium Activated Sarcoplasmic Factor (CASF) Isolation

It was apparent from the work on the biochemical and physiological parameters that amino acid mobilization had occurred and that a dramatic decrease in skeletal muscle tissue was observed. CASF has been implicated in the breakdown of myofibrillar proteins

(Busch et al., 1972; Penny, 1974; Reddy et al., 1975). Thus, the calcium activated sarcoplasmic factor may be an important enzyme in the mobilization of amino acids.

In conjunction with the fasting study CASF activity and the amount of enzyme present in the tissue were monitored. It is a primary axiom in enzymology that when studying an enzyme a source rich in the enzyme should be selected, or if limited by the source, conditions should be created that increase the amount of enzyme. Since there has been only limited work on CASF isolation from various other species, conditions for the enhancement of enzyme production were sought. All of the studies cited thus far used the back and hind limb muscles from animals under an adequate nutritional regimen. However, if this enzyme is active in protein turnover and amino acid mobilization, then the fasting state should enhance the amount of enzyme present or increase its activity. Tables 8 and 9 present data for CASF isolations from fed and fasted rabbits. The data in these tables show that there is a quantitative difference between the fed and fasted rabbits that goes beyond the disproportionate numbers in each treatment. In the first step of the isolation procedure, which involves centrifugation to clarify the supernatant, a greater yield of protein was found in the fasted treatment (1,040.8 mg protein) than in the fed treatment (384.2 mg protein). Yet the specific activity of the CASF from the fasted treatment (43.5 units/mg protein) was greater than the specific activity of the CASF from the fed treatment (34.0 units/mg protein). This would indicate a greater amount of enzyme was present in the supernatant,

TABLE 8.--Isolation data for calcium activated sarcoplasmic factor derived from muscles of ad libitum fed rabbits, n = 4.

Treatment	Volume (ml)	Total Activity (units)	Total Protein (mg)	Specific Activity (units/mg)	Fold Purification *(10^{-3})	% Recovery *(10^{-3})
Crude CASF	833	169,800	13,863	12.3	1.0	--
Centrifugation	160	13,081	384.2	34.0	*30	*80
20-40% amm. sulfate	40	2,608	84.4	31.6	*10	*20
DE 52 separation	10	515	7.7	66.9	* 1	* 3
G-200 separation	4	63.6	0.01	662.5	* 0.01	0.4

TABLE 9.--Isolation data for calcium activated sarcoplasmic factor derived from muscles of 28-day fasted rabbits, n = 8.

Treatment	Volume (ml)	Total Activity (units)	Total Protein (mg)	Specific Activity (units/mg)	Fold Purification *(10^{-3})	% Recovery *(10^{-3})
Crude CASF	1,764	339,179	27,692	12.3	1.0	--
Centrifugation	353	45,229	1,040.8	43.5	*40	*130
20-40% amm. sulfate	50	14,400	635.0	22.7	*20	* 40
DE 52 separation	30	3,756	44.2	85.0	* 2	* 11
G-200 separation	6	202	0.3	697.2	* 0.1	* 0.6

or that less non-proteolytic enzymes were present (i.e., the non-essential proteins were degraded for amino acid mobilization). There was a loss of activity in the ammonium sulfate precipitation step for both treatments. However, the fasted treatment exhibited a 48% decrease in the specific activity while the fed treatment decreased only 7%. However, during ion exchange separation the specific activity of the fasted treatment CASF increased to a value 1.3 times greater than the fed treatment CASF specific activity in spite of the fact that it yielded 6 times more protein, also.

The elution chromatograms from the ion exchange, DE 52 Cellulose, column graphically depict what Tables 8 and 9 imply. Figures 20 and 21 present the ion exchange separation for the fasted and fed treatments, respectively. Figure 21 illustrates the point that there appears to be a large amount of protein from the fed treatment that is non-proteolytic in activity. (E.g., tube 14, fed, contained greater than 120 μg/ml protein with little activity, while tube 14, fasted, contained 5.5 μg/ml protein with the same activity.) Also, the protease appears to be less active (60 enzyme units) from the fed treatment than from the fasted treatment (120 enzyme units), although this might be a reflection of the high non-proteolytic protein content of the fed treatment CASF. This result would be expected if the enzyme were participating in protein breakdown and amino acid mobilization. After 28 days the extraneous proteins should have been degraded into their constituent amino acids, therefore, there should be less protein. This situation alone would increase the specific activity of the enzyme by removing

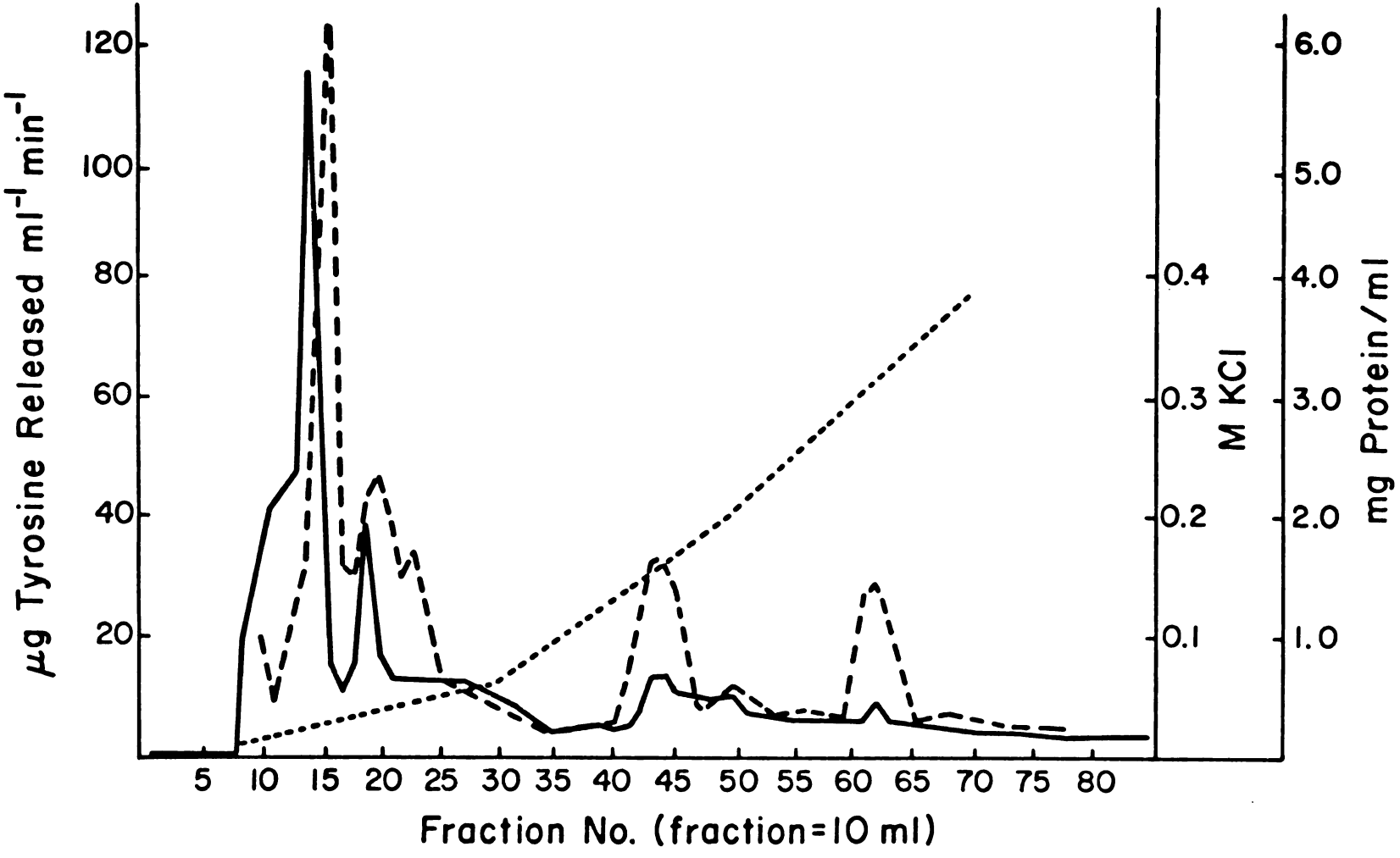


Figure 20.--DE 52 Cellulose ion exchange separation of CASF from the muscle of 28-day fasted adult male rabbits. Protein determination (———); enzyme assay (- - -); KCl gradient (-----).

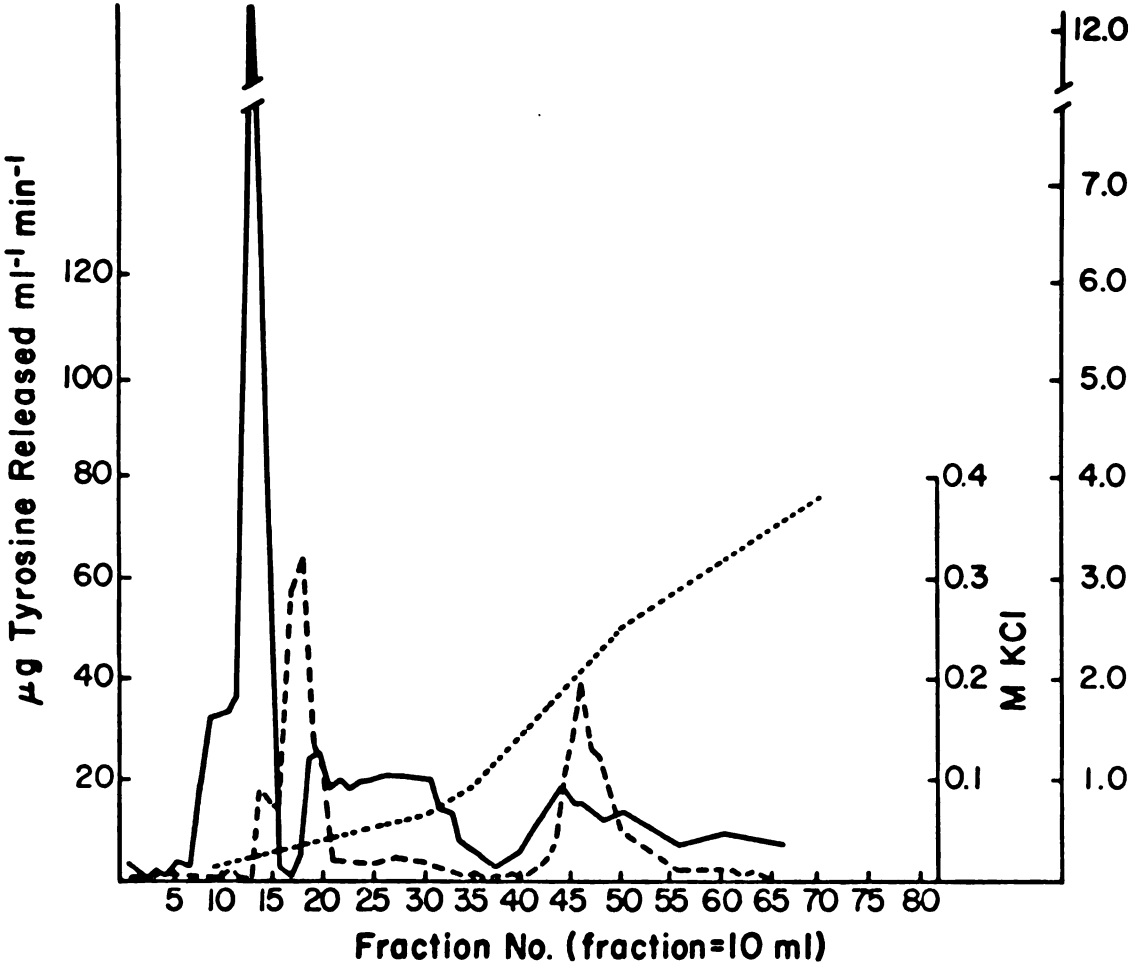


Figure 21.--DE 52 Cellulose ion exchange separation of CASF from the muscle of ad libitum fed adult male rabbits. Protein determination (———); enzyme assay (- - -); KCl gradient (------).

non-proteolytic enzymes. There is little corroborative data to cite since the only previous study fasted the rabbits for only 3.5 days (Block and Hubbard, 1962).

The Sephadex G-200 separation yielded an enzyme from both treatments with relatively the same specific activity (Tables 8 and 9). This would indicate that there was a possibility that another protease was present in the fasted system. This protease would be eliminated by G-200 separation yet carried through with CASF during the other isolation steps. A look at the DE 52 Cellulose chromatogram (Figure 20) revealed the presence of two other peaks of protease activity in the fasted muscle. The fed treatment muscle only had one other peak of protease activity. The result of the fed treatment muscle DE 52 separation (Figure 21) appeared to corroborate the result reported by Robson et al. (1974).

Catheptic Activity

Because of the nature of the study (extended fast) and the potential presence of another proteolytic enzyme as revealed in the preceding paragraphs, the catheptic activity of the preparation was assayed. The specific catheptic activity that was monitored was the hydrolytic activity of cathepsin D. The result of this determination is presented in Table 10.

It is readily apparent from Table 10 that catheptic activity was present in the preparations from both the fed and fasted samples. However, the fasted animal preparations demonstrated higher levels of catheptic activity than did the ad libitum fed animals.

TABLE 10.--Catheptic activity in calcium activated sarcoplasmic factor preparations. DE 52 Cellulose fractions were tested from the preparation by the method of Anson (1938) and Bodwell and Pearson (1963).

Preparation	Corrected O.D. at 280 nm	% Increase Over Crude Homogenate
CASF-Fed-Peak One	0.009	--
CASF-Fed-Peak Two	0.022	244
CASF-Fasted-Peak One	0.034	378
CASF-Fasted-Peak Two	0.028	311
CASF-Fasted-Peak Three	0.000	--
Crude homogenate	0.009	--

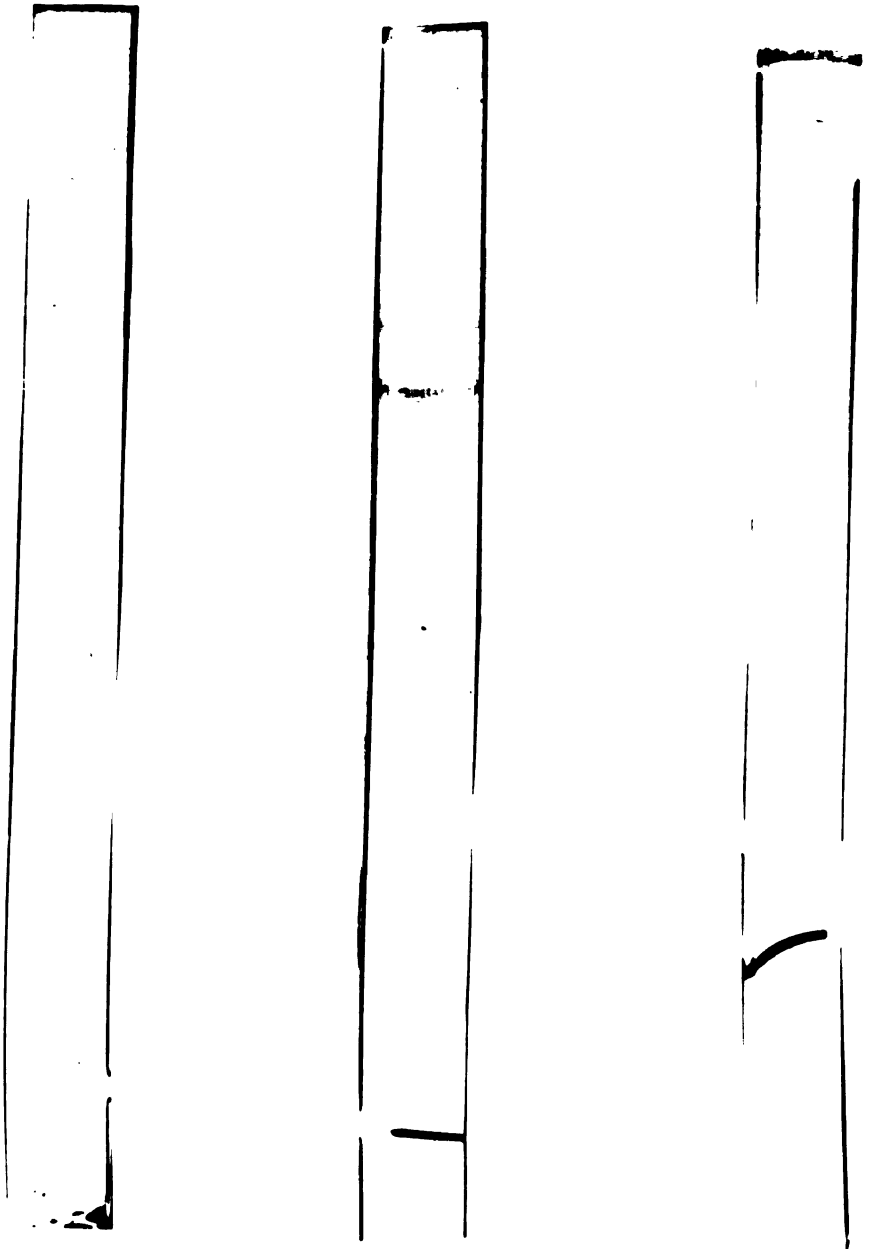
This catheptic activity may have enhanced the results of the fasted rabbit CASF isolation. Since it was possible for the presence of a second proteolytic enzyme to increase total proteolysis as measured by casein assay. The captheptic activity of G-200 peaks was not included in Table 10 or Appendix D.3.3. because the results were highly ambiguous.

SDS Polyacrylamide Gel Electrophoresis

Figure 22 depicts the result of the isolation procedure upon the CASF enzyme. Figure 23 presents the final gels for both the fed and fasted enzymes. The results for both the fasted and fed animals of molecular weight determinations by the SDS method showed single peaks at 95,000 to 100,000 and 30,000 to 35,000 daltons. This corresponds to the report of Goll et al. (1974).

Figure 22.--The results of the CASF isolation procedures on SDS electrophoresis gels.
1. Crude CASF homogenate.
2. Centrifugally clarified supernatant.
3. 20-40% ammonium sulfate cut.
4. DE 52 Cellulose separation.

Figure 23.--CASF final isolation SDS gels of G-200 separated fractions.
1. CASF from ad libitum fed rabbit muscle.
2. CASF from 28-day fasted rabbit muscle.
3. Pseudomonas perolens protease.



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Kinase Activating Factor (KAF) Isolation

Five years before the isolation of the CASF proteolytic presence in muscle tissue, the kinase activating factor (KAF) was characterized by Krebs and colleagues (Krebs et al., 1964; Meyer et al., 1964; Drummond and Duncan, 1966; Huston and Krebs, 1968). A result of this characterization was the discovery of proteolytic activity in the purified preparation. Because of the similarity of activity (proteolytic) and location (skeletal muscle) a number of investigators have mentioned the possibility that the two enzymes, CASF and KAF, may be the same (Newsholme and Start, 1973; Penny et al., 1974; Reddy et al., 1975). Therefore a tandem study involved the isolation of KAF from 16 ad libitum fed rabbits. KAF was not isolated from fasted rabbits.

Table 11 presents the data from the KAF isolation. Initially, the values reported are comparable to the values reported by Huston and Krebs (1968). However, later values are much lower than those reported by them. A partial accounting of this difference with respect to specific activity is the result of the enzyme assay. They (Huston and Krebs, 1968) reported KAF units as that amount of KAF which causes activation of phosphorylase kinase at a rate of 400 kinase units/minute as measured at pH 6.2. The results reported here refer to the proteolytic activity as determined by the casein assay. (All assays on figures will indicate proteolytic activity unless otherwise stated.)

TABLE 11.--Isolation data for the kinase activating factor from the back and hind limb muscles of adult male rabbits, n = 16.

Treatment	Volume (ml)	Total Activity (units)	Total Protein (mg)	Specific Activity (units/mg)	Fold Purification *(10^{-3})	% Recovery *(10^{-3})
Crude KAF	1,105	11,721	5,083	2.3	1.0	--
Centrifugation	829	102,916	1,746	59.0	0.34	878
TEAE-Cellulose	75	1,084	76	14.3	*20	*90
Ultrafiltration	30	6,938	100	69.5	*20	*59
Alumina C_2	16	50	0.9	55.0	* 2	* 4
G-200 separation	8	128	0.7	182.9	* 1	*11

The result of ion exchange (TEAE-Cellulose) chromatography is presented in Figure 24. The similarity between this chromatogram and that of the CASF chromatograms is very striking. However, the enzyme activity was one-half of that displayed by the CASF from ad libitum fed rabbits and one-quarter of the activity of the CASF from the fasted rabbit muscle.

The alumina C_2 absorption step yielded highly ambiguous results and should probably be excluded from the isolation procedure because of the vagaries of the material. Care was taken in this study to use "aged" alumina C but the results were still highly variable.

The G-200 Sephadex separation was very similar to reported separations (Huston and Krebs, 1968). These data are included in Figure 24. This chromatogram is similar to the G-200 Sephadex separations of the CASF factor accomplished for this study and reported in the literature (Robson et al., 1974).

The G-200 separated material also yielded highly ambiguous results during catheptic enzyme assay. KAF preparations did not show catheptic activity.

Phosphorylase Kinase Activation

The ability to activate phosphorylase kinase corresponded to the G-200 peak encompassing tubes 14-16, in Figure 24. The activity reported by Huston and Krebs (1968) was 3.8 KAF units/ml, while the value for this study was 3.7 KAF units/ml. A KAF unit was defined by these authors as that amount of KAF which causes activation of phosphorylase kinase at a rate of 400 kinase units/minute.

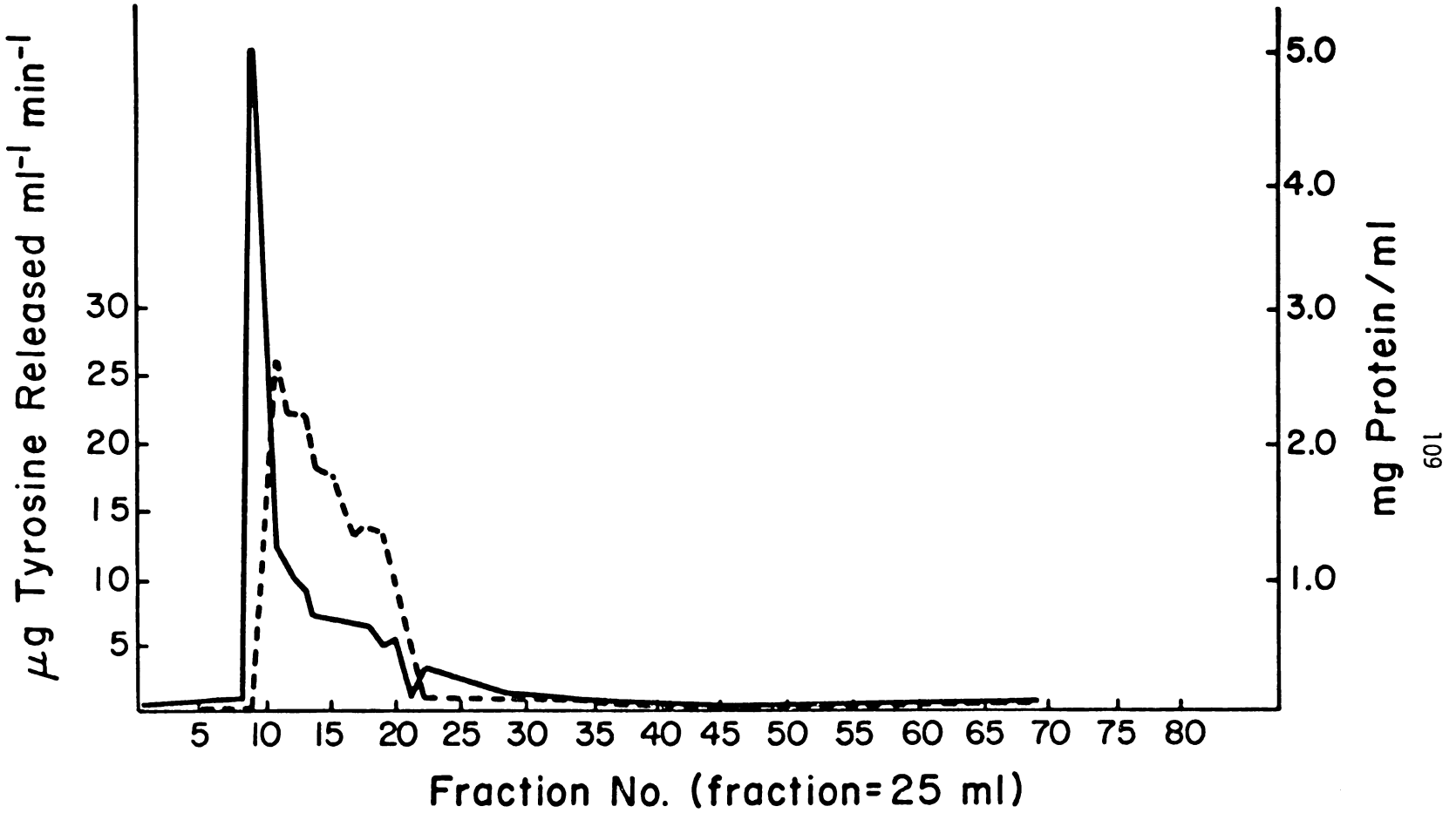


Figure 24.--Separation of kinase activating factor via TEAE-Cellulose ion exchange chromatography. Solid line (———) represents protein determination and dashed line (- - -) represents enzyme activity.

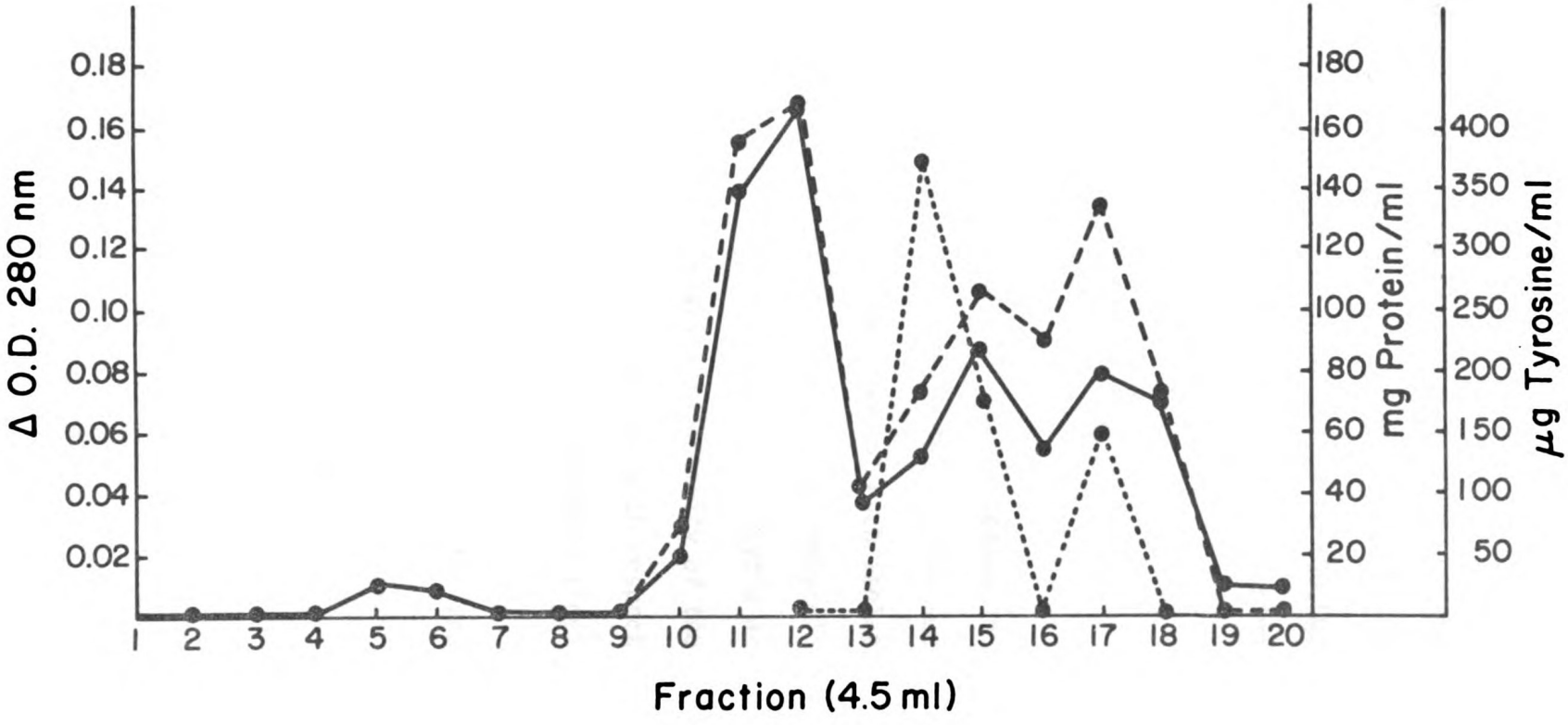


Figure 25.--G-200 Sephadex separation of kinase activating factor. Solid line indicates O.D. at 280 nm (———); dashed line indicates protein as determined by fluorescamine (- - -); dotted line indicates enzyme activity (------).

SDS Polyacrylamide Gel Electrophoresis

The isolation procedure is depicted by SDS gels in Figure 26. The molecular weights of KAF as determined by SDS gel electrophoresis yields a major fraction at 95,000 to 100,000 and a smaller fraction 30,000 to 35,000 daltons. This is very similar to the results reported for the CASF enzyme.

Comparative Enzyme Activity

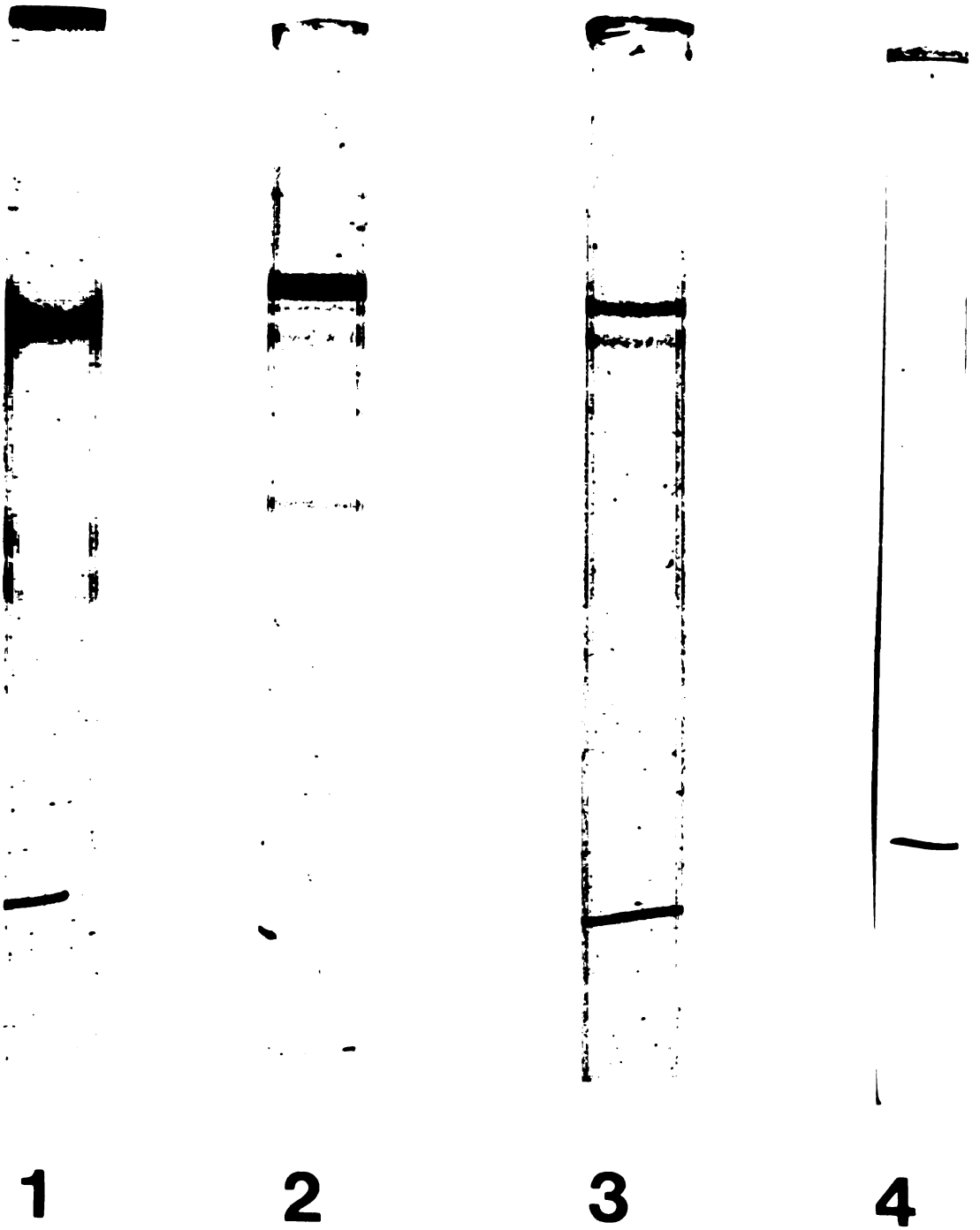
Proteolytic Activity

The proteolytic activity of the proteases in this study was assayed using casein as a substrate. The enzymes used for the rest of this study are the pooled fractions of each isolation procedure that demonstrated the highest activity after molecular exclusion chromatography. Table 12 compares the proteolytic activity of the various proteases. The similarity in activity of the KAF and CASF from the ad libitum fed animals was less with respect to specific activity, because the KAF fractions possessed 3 times more protein

TABLE 12.--Comparative proteolytic activities for proteases of different origin.

Protease	Enzyme Units	Specific Activity (units/mg)
CASF (Fed)	15.9	662.5
CASF (Fasted)	67.4	686.8
KAF	14.6	197.3
Ps. perolens protease	480.8	4,370.9

Figure 26.--SDS gel electrophoresis of isolation procedure.
1. Crude KAF.
2. Centrifugally clarified KAF.
3. TEAE-Cellulose separation.
4. G-200 Sephadex separation.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than did the CASF (fed) fraction. The protease from Ps. perolens demonstrated the greatest amount of proteolytic activity. The activity of this bacterial protease, although high, would not approach the activity of a digestive enzyme such as trypsin. Huston and Krebs (1968) demonstrated that trypsin in a similar environment could show a specific activity of 73,000 units/mg protein. However, all of the above enzymes possessed at least a modicum of proteolytic activity (Appendix E.1.1).

Phosphorylase Kinase Activating Activity

A number of investigators became involved with KAF because of its ability to "short circuit" the enzyme cascade system depicted in Figure 1 and activate phosphorylase kinase. Newsholme and Start (1973) and Huston and Krebs (1968) postulated that this "short circuiting" was the result of proteolysis. The mechanism involved the release of the catalytic subunit from the regulatory subunit by proteolytic cleavage.

Table 13 presents the comparative ability of the proteases to activate phosphorylase kinase. The value for KAF was very low in comparison to values reported earlier in this study. The CASF (Fed) value is one unit/ml higher than the earlier reported value for KAF. The CASF (Fasted) enzyme had the highest value among all of the proteases in this study. Apparently the protease from Ps. perolens does little to activate the enzyme. More likely, it completely degrades the substrate. Apparently, the activation of phosphorylase kinase involves the separation of the regulatory and catalytic subunits by

TABLE 13.--Phosphorylase kinase activating activity of proteases from various origins.

Protease	KAF Units*
CASF (Fed)	4.93
CASF (Fasted)	6.23
KAF	1.83
Ps. perolens protease	1.99

*Units defined in text.

cleavage. This was demonstrated by Huston and Krebs (1968) who demonstrated that trypsin and chymotrypsin activated phosphorylase kinase equally but 3 times greater than KAF.

Synthetic Substrate Hydrolytic Activity

All of the vertebrate proteases responded very poorly to both synthetic substrates and presented no clear-cut results. Apparently, neither synthetic substrate was the proper dipeptide for hydrolysis.

Inhibition by Bovine Heart KAF Inhibitor

Drummond and Duncan (1966) reported the isolation of a factor which prevented calcium activation by KAF. This factor was isolated from bovine hearts. The inhibitory factor appeared to work stoichiometrically rather than catalytically upon the activating factor.

This factor (inhibitor) was isolated from bovine hearts and tested upon the proteases of this study (Appendix E.1.3). The results of this study are presented in Table 14. All three vertebrate enzymes were substantially inhibited by the presence of bovine heart KAF inhibitor. The bacterial enzyme was not inhibited to any substantial degree.

TABLE 14.--The effect of bovine heart KAF inhibitor upon the proteolytic activity of various proteases.

Protease	Enzyme Activity (units)		% Inhibition
	No Inhibitor	Inhibitor	
CASF (Fed)	7.0	0.6	91.4
CASF (Fasted)	31.5	4.4	86.0
KAF	6.7	0.0	100.0
Ps. perolens protease	530.2	509.6	3.9

Activity on Myofibrils

Phase contrast microscopy.--The proteases isolated in this study were introduced into a myofibrillar system. This system was described by Busch et al. (1972) and Goll et al. (1974) (Appendix F.1.1). The ratio of substrate (myofibrils) to enzyme was 5,000:1. This ratio was higher than that described by Etlinger and Fishman (1973) who used a ratio of 2,000:1. The samples were viewed 24 hours after incubation on a Zeiss photo-microscope III.

Figure 27 illustrates the appearance of the control sample of myofibrils for either the EDTA or $CaCl_2$ added samples. There appeared to be a few myofibrils in the samples with 10 mM $CaCl_2$ added that showed some Z-line breakdown. This perhaps could be attributed to intrinsic catheptic enzymes released during myofibril preparation.

Figures 28 and 29 demonstrate the effect of CASF (Fed) and CASF (Fasted) upon the myofibrils, respectively, as observed under phase contrast conditions. There was no apparent difference between the CASF samples. Both CASF types demonstrated Z-line degradation. Z-line degradation assumed two forms: either a "splotchy" Z-line appearance (Figure 28) or the complete removal of Z-lines (Figure 29). The sample with 10 mM $CaCl_2$ added to the incubation mixture demonstrated these effects. Some myofibrils in the 10 mM EDTA added to the incubation mixture demonstrated these effects but not to the extent shown in the $CaCl_2$ added samples.

The effect of KAF addition to the myofibril incubation mixture yielded the same results. These results are depicted in Figures 30 and 31. Some fragmentation (Figure 31) occurred, however, this may have been the result of mechanical handling. There was no observable difference between the KAF and CASF activities on the myofibrils.

The addition of the Ps. perolens protease to the myofibril mixture resulted in extensive degradation of the myofibril. Figure 32 demonstrates the release of Z-line material, some fragmentation

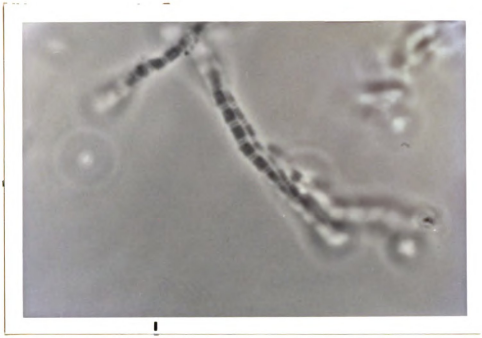


Figure 27.--Myofibril:enzyme mixture with $CaCl_2$ (10 mM) added, control. (Magnification 1,250X.)

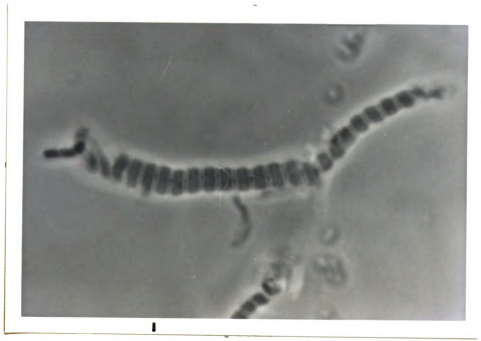


Figure 28.--Myofibril:enzyme (CASF-Fed) mixture with $CaCl_2$ (10 mM) added. (Magnification 1,250X.)

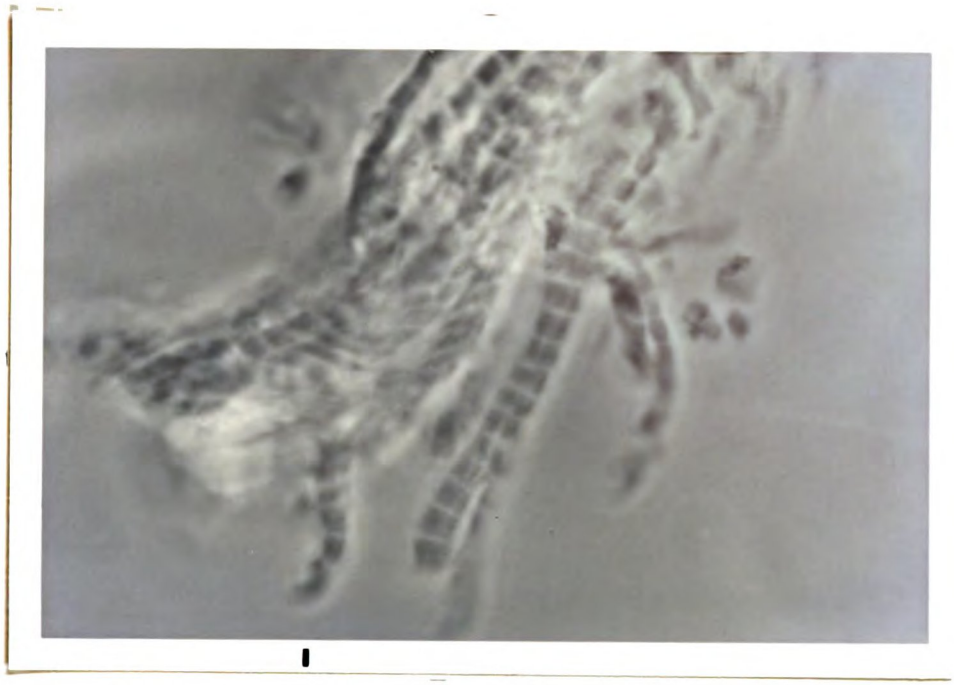


Figure 29.--Myofibril:enzyme (CASF-Fasted) mixture with $CaCl_2$ (10 mM) added (Magnification 1,250X.)

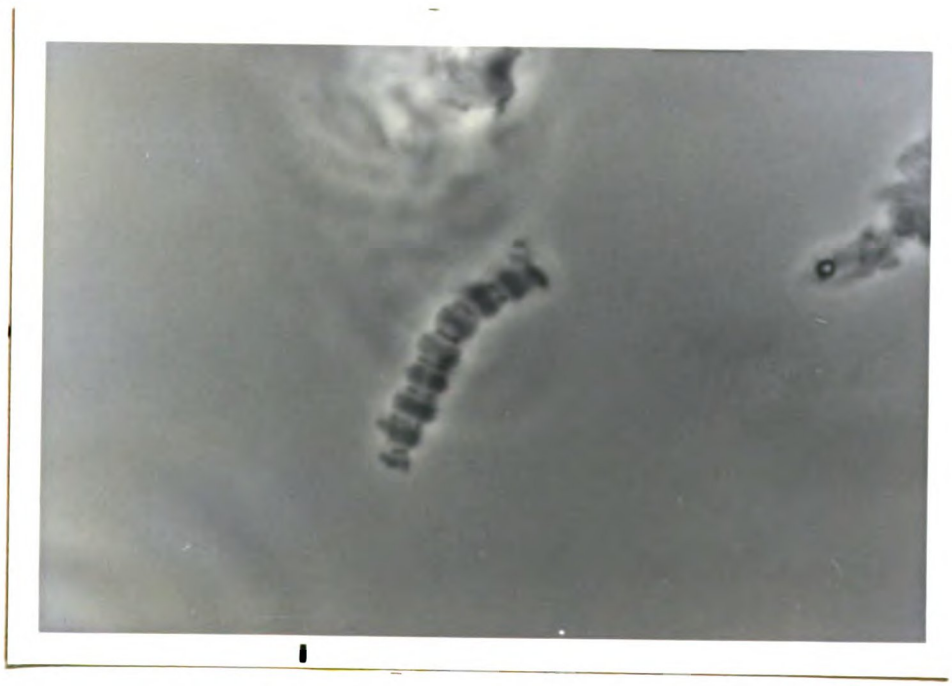


Figure 30.--Myofibril:enzyme (KAF) mixture with $CaCl_2$ (10 mM) added. (Magnification 1,250X.)

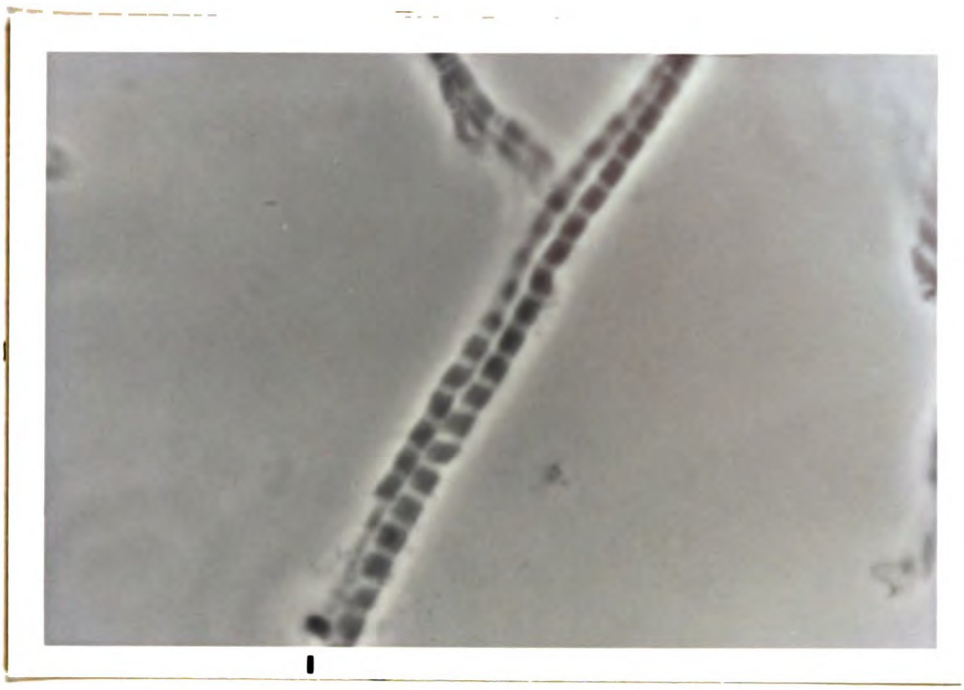


Figure 31.--Myofibril:enzyme (KAF) mixture with $CaCl_2$ (10 mM) added. (Magnification 1,250X.)

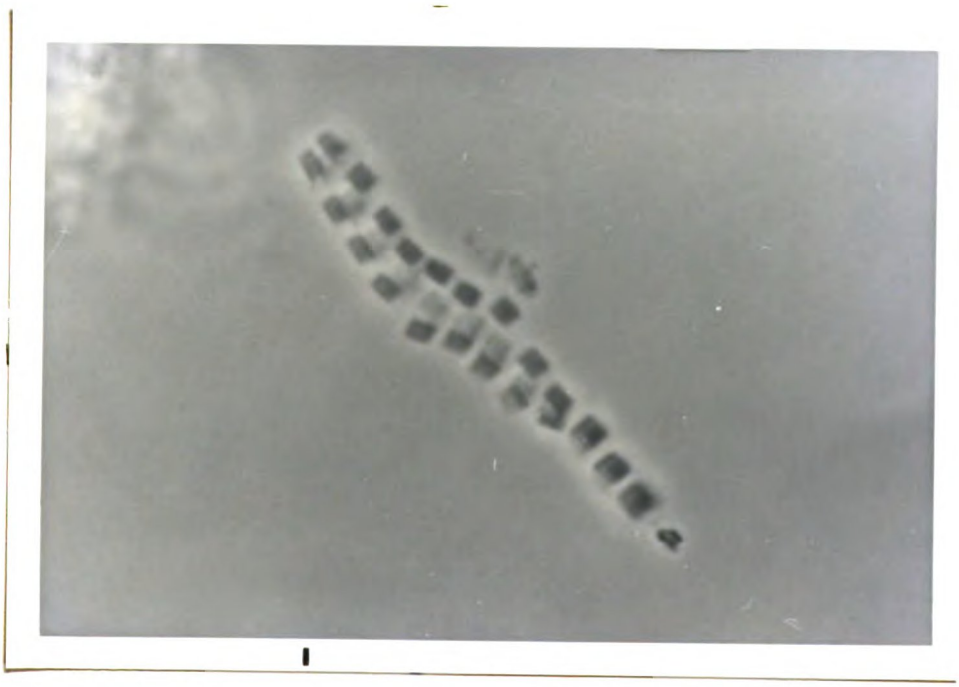


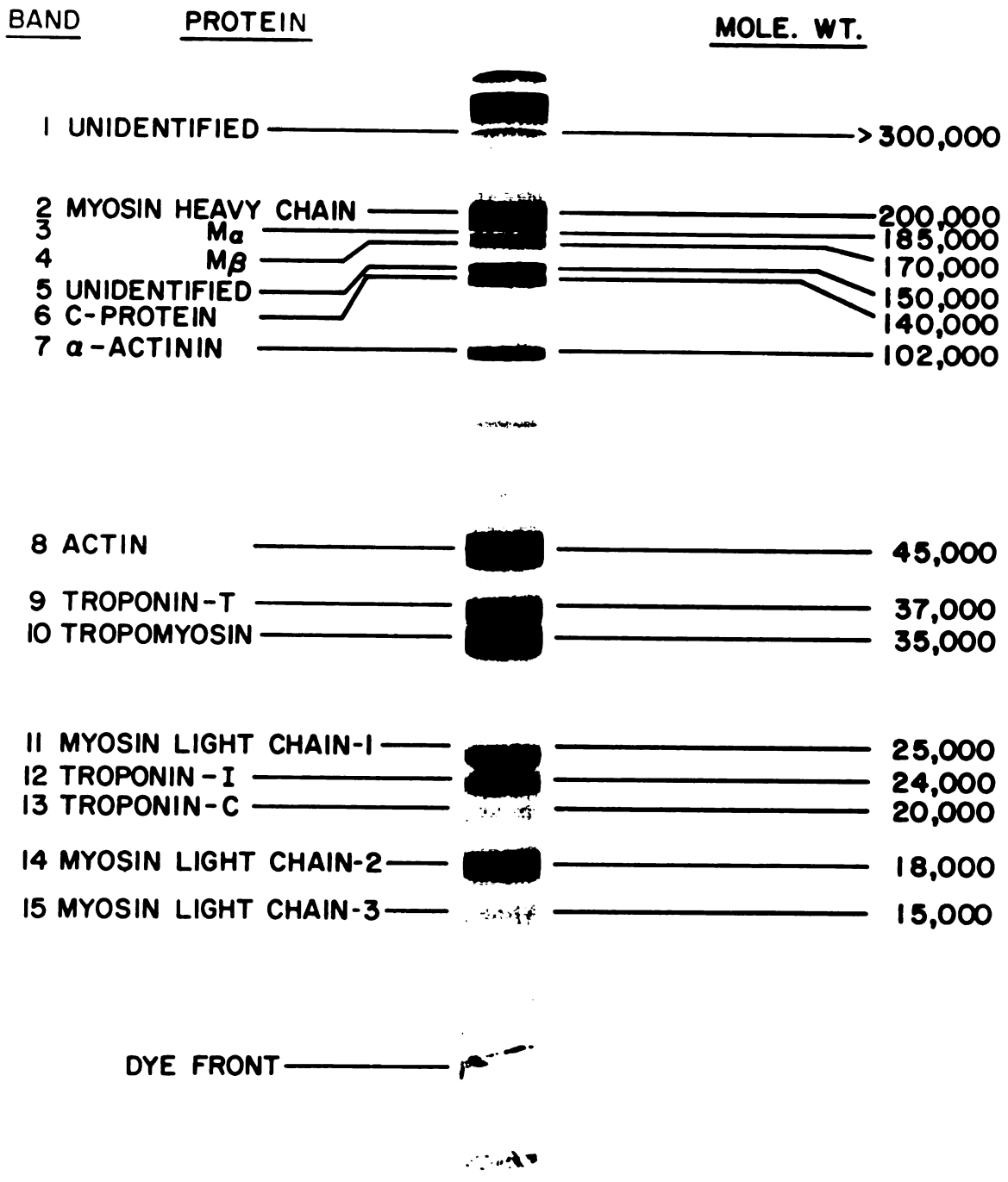
Figure 32.--Myofibril:enzyme (Ps. perolens protease) mixture with $CaCl_2$ (10 mM) added. (Magnification 1,250X.)

of the sarcomeres, but the myosin (A band) components appear to still be in register within the myofibril.

SDS polyacrylamide gel electrophoresis of myofibrils.--Figure 33 will serve as a legend for this discussion on the effect of the proteases upon myofibrillar protein integrity. A number of investigators have reported work on myofibrillar degradation by various proteases, or CASF (Penny, 1974; Reddy et al., 1975; Robson et al., 1974). This has been an area of interest since the phenomenon of "rigor resolution" has been attributed to Z-line degradation by CASF (Goll et al., 1974). They postulated that since only α-actinin, troponin and tropomyosin are digested by CASF, Z-line degradation is the result of the loss of integrity of α-actinin in the Z-line (Goll et al., 1974; Penny, 1974; Reddy et al., 1975). No one has reported the degradation of myosin by CASF or KAF. This is the pivotal point since α-actinin, troponin and tropomyosin only account for 12% of the myofibril by weight (Goll et al., 1974). Far more mobilization of amino acids and protein degradation takes place than can be accounted for with these proteins. This was apparent from the fasting study cited earlier.

Purified bacterial proteases are capable of myosin degradation (Porzio, 1976). However, the intrinsic proteases of skeletal muscle, KAF and CASF, have demonstrated only limited proteolytic ability. The catheptic enzymes are limited by location (sacs) or milieu from participation in protein turnover. Although Moeller et al. (1976) have restored catheptic activity as the predominant

Figure 33.--Sodium dodecyl sulfate polyacrylamide gel electrophoresis gel of myofibril preparation. Molecular weights and band assignments from Porzio, 1976.


BAND
PROTEIN
MOLE. WT.
1 UNIDENTIFIED
>300,000
2 MYOSIN HEAVY CHAIN
200,000
3 Mα
185,000
4 Mβ
170,000
5 UNIDENTIFIED
150,000
6 C-PROTEIN
140,000
7 α-ACTININ
102,000
8 ACTIN
45,000
9 TROPONIN-T
37,000
10 TROPOMYOSIN
35,000
11 MYOSIN LIGHT CHAIN-1
25,000
12 TROPONIN-I
24,000
13 TROPONIN-C
20,000
14 MYOSIN LIGHT CHAIN-2
18,000
15 MYOSIN LIGHT CHAIN-3
15,000
DYE FRONT

activity in high temperature conditioning of carcasses. They implicated cathepsin C.

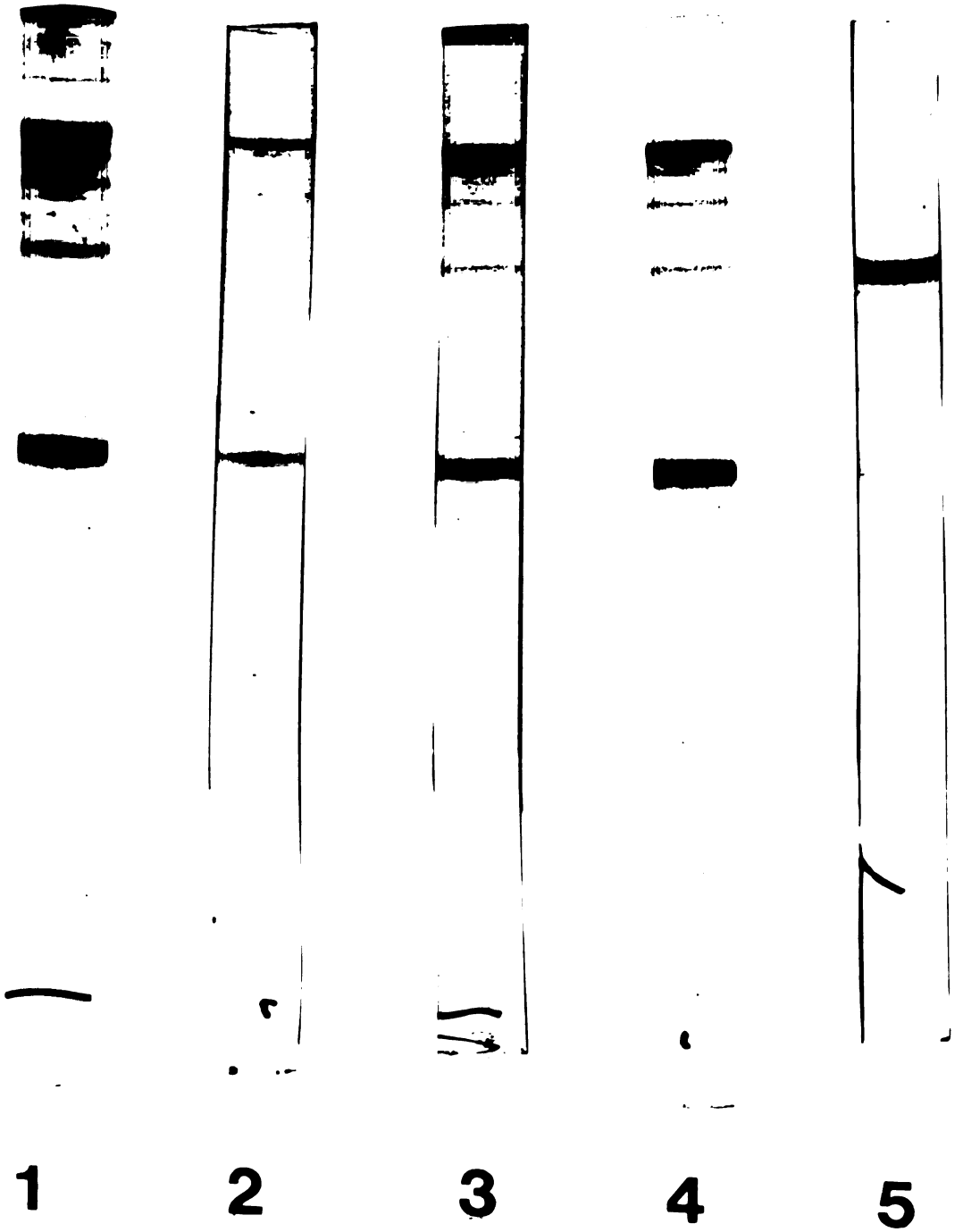
Figure 34 presents the gels of myofibrils exposed to the various proteases and activated by 10 mM $CaCl_2$. The lessening of intensity of the α-actinin band was apparent. The effect of the proteases (intrinsic) upon the troponins and tropomyosin were less apparent. There was little difference between the CASF and KAF activity on the myofibril as was indicated by the phase contrast microscope study. The number of small bands beneath the myosin heavy chain appears to be the same, thus no myosin digestion was noted. The above results were in direct contrast to the effect of the Ps. perolens protease upon the myofibril. Extensive degradation was apparent from the gel which corroborates the phase contrast microscope study. There was a heavy accumulation of light chain elements in the lower portion of the gel. A similar effect was reported by Porzio (1976) and Reddy et al. (1975), using a Ps. fragi protease and trypsin, respectively.

Gel electrophoresis of myofibrils incubated with 10 mM EDTA resembled the control gel shown in Figure 34.

Gels with bovine heart KAF inhibitor added yielded highly ambiguous results. This was a direct result of the impure nature of the inhibitor. The inhibitor bands complicated the myofibril structure beyond usefulness.

In spite of the above ambiguous results with the bovine heart KAF inhibitor, a comparison of KAF and CASF activity on myofibrillar tissue, as observed microscopically and electrophoretically, in

Figure 34.--SDS gel electrophoresis of myofibrils incubated with various proteases.
1. Control.
2. CASF (Fed).
3. CASF (Fasted).
4. KAF.
5. Ps. perolens protease.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conjunction with the KAF inhibitor study demonstrated a similarity between these two enzymes. The SDS gel electrophoresis of KAF and CASF and their activity on casein and phosphorylase kinase demonstrated this similarity also, especially when these two enzymes were compared to any of the Pseudomonas perolens protease results.

V. SUMMARY

Exogenous (Bacterial) Protease

To facilitate the study of the extracellular protease isolated from Pseudomonas perolens ATCC 10757 by Buckley (1972), growth and enrichment of the organism was necessary. Growth, as monitored by protein determination, was enhanced by increasing the density of the innoculum in the growth flask and by enrichment of the medium. The medium was enriched by substituting a casamino acid medium for the Koser's citrate medium used by Buckley (1972). Growth and enzyme production were increased through the addition of 10^{-5} M $ZnCl_2$ to the growth medium as well as 4.5×10^{-3} M $CaCl_2$.

The method of enzyme isolation was improved to avoid the high early losses of activity originally encountered. Because ultrafiltration resulted in the largest decrease in enzyme activity it was eliminated. DEAE-Sephadex A-50 batch absorption, ammonium sulfate precipitation (50% saturation) and Sephadex G-100 molecular exclusion chromatography were substituted for the ultrafiltration step. This procedure yielded 0.01% protein recovery but a 967 fold purification of enzyme.

The pH optima, temperature optima and ability to be inhibited by particular inhibitors corroborated the work of Buckley (1972). However, the protease isolated by the above described method was at least 50 times more sensitive to EDTA than earlier reported (Buckley,

1972). Restoration of activity through $CaCl_2$ addition was never complete.

The kinetics of hydrolysis were obtained on N-CBZ-glycyl-L-leucine. The protease had a V_{max} and K_m of 169.5 μM leucine ml^{-1} min^{-1} and 2.6 mM, respectively. Results substantiate the hypothesis of Buckley (1972) concerning the action of this protease on collagen.

SDS gel electrophoresis disclosed a molecular weight of 30,000 to 35,000 daltons.

Endogenous Muscle Proteases

Calcium Activated Sarcoplasmic Factor

To ascertain the feasibility of increasing CASF production or activity in rabbit skeletal muscle, animals were fasted for 28 days. The effects of fasting were monitored by live animal weight, serum glucose levels, serum nonesterified free fatty acid levels and serum total free amino acid levels. The weights of two muscles (semitendinosus and longissimus) and any attendant changes in total, myofibrillar, sarcoplasmic and stromal protein and non-protein fractions were determined also. The values obtained from the fasted rabbits were compared against control, ad libitum fed rabbits. Additionally, the activity and quantity of CASF from each treatment were compared.

The results were as follows:

(1) Live animal weight and average daily gain decreased and became negative, respectively, when fasted animals were compared to ad libitum fed animals.

(2) Blood glucose and total free amino acid levels in the serum were essentially the same for both treatments.

(3) Nonesterified free fatty acid levels were higher in the serum of fasted rabbits than for serum taken from ad libitum fed rabbits.

(4) The total protein, muscle weights and all of the individual protein components of muscle were higher for the ad libitum fed than for the fasted rabbits.

(5) Organ weights, heart and liver were also higher in the ad libitum fed rabbits.

(6) On an equivalent basis, the fasted rabbits yielded 15 times more enzyme with a slightly higher specific activity than the fed rabbits.

(7) Greater catheptic activity was encountered in the DE 52 Cellulose fractions of CASF derived from the fasted rabbits than the fed rabbits.

(8) SDS gel electrophoresis exhibited two components of 95,000-100,000 and 30,000-35,000 daltons.

Kinase Activating Factor

This enzyme was isolated to compare its properties with those of the calcium activated sarcoplasmic factor since both are endogenous skeletal muscle proteases found in the sarcoplasm. KAF demonstrated proteolytic activity, an ability to activate phosphorylase kinase, very little catheptic activity and molecular weights, as revealed by SDS gel electrophoresis, similar to CASF.

Comparative Enzyme Activity

The two endogenous enzymes of skeletal muscle (CASF and KAF) were compared to the bacterial protease with respect to activity on various substrates. The following results were obtained:

(1) Proteolytic activity: The protease from Ps. perolens possessed the highest specific activity on casein substrate. CASF isolated from the muscles of fasted rabbits had a specific activity less than the Ps. perolens protease but slightly higher than the CASF from fed rabbits. KAF had the lowest specific activity.

(2) Phosphorylase kinase activiation ability: The proteases in descending order of activity were CASF (Fasted), CASF (Fed), KAF and Ps. perolens protease.

(3) N-CBZ-glycyl-L-leucine hydrolysis activity: Only Ps. perolens protease exhibited this ability.

(4) Inhibition by bovine heart inhibitor of KAF: KAF was completely inhibited, followed very closely by CASF (Fasted) and CASF (Fed), respectively. The Ps. perolens protease was not inhibited.

(5) Myofibril integrity disruption ability: All of the proteases demonstrated an ability to remove the Z-disc from myofibrils. There was little or no observable difference in activity between KAF and CASF (Fasted and Fed). The Ps. perolens protease caused extensive degradation of the entire myofibrillar structure, as observed under phase contrast microscopy. SDS gel electrophoresis of the reaction mixtures corroborates the microscopic study. The KAF and CASF fractions demonstrated the loss of α-actinin and

troponin with a slight increase in lower molecular weight bands. The Ps. perolens gel demonstrated extensive proteolysis with a major reduction in larger molecular weight components and an increase in lower molecular weight fractions.

It was found that the fasting state did not increase CASF production or activity of the isolated enzyme, significantly. The physiological and biochemical changes that occurred in the fasting study could not be accounted for by either CASF or KAF enzymes. The changes that occurred were rather extensive, yet neither enzyme displayed the ability to initiate changes beyond degradation of α-actinin and tropomyosin. The bacterial protease caused extensive myofibrillar degradation, but its presence in vivo is difficult to rationalize.

A comparison of KAF and CASF activity on myofibrillar tissue, as observed microscopically and electrophoretically, in conjunction with the KAF inhibitor study demonstrated a similarity between these two enzymes. The SDS gel electrophoresis of KAF and CASF and their activity on casein and phosphorylase kinase demonstrated this similarity also, especially when these two enzymes were compared to any of the Pseudomonas perolens protease results.

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APPENDIX

Appendix A.1.1.--Fluorescamine assay and standard curve.

Standard µg/ml	Transmission	Transmission % Corrected
200	+100.0	94.5
100	57.0	52.5
50	31.5	27.0
20	15.5	11.0
10	10.5	5.5
5	7.5	3.0
1	4.5	0.0
Blank	4.5	--

Assay system: 0.5 ml Sample of Standard (BSA)
(Sample diluted 1:2)
1.0 ml 0.2 M Sodium borate (pH 9.25)
0.5 ml Fluram reagent.

Fluorimeter: Excitation, 390 nm; emission, 480 nm.

Appendix A.1.2.--Conductivity bridge of KCl gradient determination using the YSI model 31 Conductivity Bridge, Pyrex electrode (7740), K = 1.0.

Standard M KCl	x 10^4 µM Ohms	Standard M KCl	x 10^4 µM Ohms
0.00	0.43	0.30	3.20
0.05	0.87	0.40	3.80
0.10	1.42	0.60	5.00
0.20	2.10	0.80	6.40
0.25	2.50		

Standards: 0.1 M Tris-Acetate, 0.002 M EDTA, 0.001 M Thioglycollic acid, 0.001 M Sodium azide and 0.0 to 0.8 M KCl (pH 7.5).

Appendix B.1.1.--The effect of substitution of either carbon source or nitrogen source upon the ability of Pseudomonas perolens ATCC 10757 to produce an extracellular protease in substituted Koser's citrate medium, grown at 10°C (pH 7.5).

Substitution	Lowry Protein Determination			
	Sample Vol. (ml)	O.D. 660 nm	µg Protein / Test Tube	mg Protein Total
Control	1.0	0.059	30.2	6.65
Glucose	0.5	0.090	38.5	16.9
Glucose	1.0	0.174	74.4	16.4
Ammonium citrate	0.5	0.077	32.9	14.5
Ammonium citrate	1.0	0.120	51.3	11.3
Glucose, 2X	1.0	0.314	134.2	30.9
Glucose, 2X	1.0	0.280	119.2	27.5

Conditions: Supernatant of the growth medium examined by standard Lowry procedure.

Substitution	Enzyme Assay				
	Sample Vol. (ml)	O.D. 660 nm	µg Tyr.rel. / Test Tube	µg Tyr.rel. / ml/min	Total Activity
Control	1.0	0.030	3.33	0.22	48.4
Glucose	1.0	0.108	11.10	2.96	681.0
Glucose	1.0	0.106	10.70	2.92	672.0
Ammonium citrate	1.0	0.315	39.00	10.40	2,392.0
Ammonium citrate	1.0	0.270	31.90	8.52	1,960.0
Glucose, 2X	1.0	0.000	--	--	--
Glucose, 2X	1.0	0.000	--	--	--

Conditions: Supernatant of the growth medium examined by Kunitz (casein) digestion procedure.

Growth medium: See Appendix B.1.2.

Appendix B.1.2.--Substituted media used in the growth experiment cited in Appendix B.1.1. Values in grams/liter.

	Koser's Citrate	Glucose	2X Glucose	Ammonium Citrate
Calcium chloride	0.5 (4.6)	0.5 (4.6)	0.5 (4.6)	0.5 (4.6)
Magnesium sulfate	0.2 (1.7)	0.2 (1.7)	0.2 (1.7)	0.2 (1.7)
Sodium phosphate (monobasic)	1.0 (8.4)	1.0 (8.4)	1.0 (8.4)	1.0 (8.4)
Sodium ammonium phosphate	1.5 (9.7)	1.5 (9.7)	3.0(19.4)	--
Sodium citrate	3.0(11.5)	--	--	--
D-glucose	--	2.1(11.6)	4.2(23.2)	--
Ammonium citrate	--	--	--	2.2 (9.7)

1. The number in parentheses represents the millimoles of that component present in the medium (e.g., Koser's citrate, calcium chloride concentration is 4.6 millimoles).

2. All media double-distilled, deionized water, autoclaved and innoculated with a 2% (v/v) innoculum.

Appendix B.2.1.--Growth of Pseudomonas perolens in Koser's citrate medium, a protein medium and casamino acid medium. All at pH 7.5 and grown at 10°C, with and without 10^{-5} M $ZnCl_2$. Values in µg protein/ml.

Medium	$ZnCl_2$ 10^{-5}M	Protein Concentration						
		24 hr	36 hr	48 hr	60 hr	72 hr	96 hr	120 hr
Koser's citrate	-	4.9	11.7	8.8	14.1	14.7	14.7	15.0
Koser's citrate	+	13.2	15.2	12.3	19.8	19.7	12.3	17.4
Protein basal	-	7.4	7.4	7.4	29.6	6.2	8.9	7.6
Protein basal	+	1.0	5.8	12.1	13.8	7.3	8.8	9.5
Casamino acids	-	1.0	13.4	46.0	37.7	54.7	43.9	61.9
Casamino acids	+	1.0	24.9	82.0	135.2	123.8	115.8	123.2

1. Protein determined by the Lowry method.
2. Protein concentrations shown were corrected for initial protein content if applicable.

Media used in the study cited above. Values in grams/500 ml.

Media	Koser's Citrate	Protein Basal	Casamino Acids
Calcium chloride	0.25	0.25	0.25
Magnesium sulfate	0.10	0.10	--
Sodium phosphate, monobasic	0.50	0.50	--
Sodium ammonium phosphate	0.75	--	--
Sodium citrate	1.50	--	--
Hammerstein's casein	--	0.20	--
Casamino acids	--	--	2.25

1. All media were made to one-half liter quantities with deionized, double-distilled water and adjusted to pH 7.5.

2. Zinc chloride was added to a concentration of 0.00001 M where it was signified in the above table.

Appendix B.2.2.--Enzyme activity monitored in the medium innoculated with Pseudomonas perolens. Media consisted of Koser's citrate, protein basal medium and casamino acid medium, adjusted to pH 7.5 and grown at 10°C with constant agitation.

Medium	$ZnCl_2$ $10^{-5}M$	Enzyme Activity, µg tyrosine released/ml per minute						
		24 hr	36 hr	48 hr	60 hr	72 hr	96 hr	120 hr
Koser's citrate	-	0.3	1.1	0.9	0.6	1.0	0.7	0.7
Koser's citrate	+	1.0	0.8	1.2	1.0	0.9	0.5	0.3
Protein basal	-	0.2	0.7	2.0	0.9	0.9	0.2	0.3
Protein basal	+	0.3	0.7	0.7	0.5	0.6	1.0	0.8
Casamino acids	-	0.0	1.0	0.8	1.8	0.0	0.0	0.0
Casamino acids	+	0.3	1.8	2.9	3.1	3.5	2.9	1.1

1. Protein determined by Lowry method and presented in Appendix B.2.1.
2. Enzyme assay: Kunitz assay, casein proteolysis.
3. Media preparation: see also Appendix B.2.1.

Appendix B.2.3.--Effect of varying Zn^{++} concentrations upon the growth and enzyme production by Pseudomonas perolens ATCC 10757 on Koser's citrate and protein basal medium.

A. Protein Determination

Medium	Zn^{++} 10^{-9} M	µg Protein/ml		
		12 hr	24 hr	48 hr
Protein basal	0	4.3	6.8	1.3
Protein basal	48,000	12.0	17.3	3.3
Protein basal	480	4.3	6.9	2.0
Protein basal	4.8	3.0	7.0	2.0
Koser's citrate	0	4.8	12.3	3.0
Koser's citrate	48,000	15.0	22.3	4.6
Koser's citrate	480	4.9	12.1	3.0

B. Enzyme Production and Activity

Medium	Zn^{++} 10^{-9} M	Enzyme Units		
		12 hr	24 hr	48 hr
Protein basal	0	0.05	0.07	0.06
Protein basal	48,000	0.12	0.04	0.09
Protein basal	480	0.01	0.00	0.00
Protein basal	4.8	0.11	0.01	0.00
Koser's citrate	0	0.05	0.01	0.11
Koser's citrate	48,000	0.11	0.00	0.14
Koser's citrate	480	0.06	0.00	0.10

Media prepared according to Appendix B.2.1.

Appendix C.1.1.--Ascertaining the pH and ionic strength necessary to elute Pseudomonas perolens ATCC 10757 extracellular protease from DEAE-Sephadex A-50.

Eluent	Protein		Enzyme Activity			Spec. Act.
	O.D. 660 nm	µg Protein / ml Eluent	O.D. 660 nm	µg Tyr. / ml	Units	Units / mg Protein
pH 5.0	0.070	25.5	0.000	0.00	0.00	0.00
pH 5.0	0.070	25.5	0.000	0.00	0.00	0.00
pH 6.0	0.075	27.8	0.001	0.09	0.02	0.75
pH 6.0	0.070	25.5	0.001	0.09	0.02	0.78
pH 8.0	0.082	30.3	0.048	4.33	1.15	39.70
pH 8.0	0.076	28.1	0.051	4.60	1.22	41.90
pH 9.0	0.086	31.8	0.030	2.70	0.72	23.80
pH 9.0	0.079	38.7	0.051	4.60	1.22	40.30
200 mM Tris	0.062	22.8	0.012	1.08	0.29	13.50
200 mM Tris	0.055	20.3	0.005	0.45	0.12	5.60
400 mM Tris	0.069	25.4	0.007	0.63	0.17	6.80
400 mM Tris	0.066	24.4	0.011	0.99	0.26	10.40
600 mM Tris	0.104	38.4	0.011	0.99	0.26	6.90
600 mM Tris	0.100	36.9	0.013	1.17	0.31	8.20
800 mM Tris	0.073	26.9	0.036	3.24	0.86	26.10
800 mM Tris	0.106	39.1	0.042	3.78	1.01	30.60
1,000 mM Tris	0.123	45.5	0.029	2.61	0.70	15.80
1,000 mM Tris	0.118	43.5	0.033	2.97	0.79	17.80

All eluents were 0.1 M Tris-HCl, 0.0045 M $CaCl_2$ (pH 7.0) unless ionic strength or pH is listed differently in the table.

Appendix C.1.2.--The efficacy of sodium chloride as a substitute for Tris-HCl in the DEAE-Sephadex A-50 eluent.

Eluent	Protein		Enzyme Activity			Spec. Act.
	O.D. 660 nm	µg Protein / ml Eluent	O.D. 660 nm	µg Tyr. / ml	Units	Units / mg Protein
Initial supernatant	0.671	144.9	0.281	100.8	6.72	
Initial supernatant	0.652	140.2	0.283	101.6	6.75	47.4
DEAE-eluate 800 mM Tris	0.218	46.9	0.057	20.4	1.36	
DEAE-eluate 800 mM Tris	0.219	46.9	0.063	20.8	1.52	30.7
800 mM NaCl	0.495	106.6	0.324	116.4	7.76	
800 mM NaCl	0.476	102.4	0.296	106.4	7.09	71.2
Ammonium sulfate pptn. Tris eluate	0.062	133.3	0.021	75.2	5.01	
Ammonium sulfate pptn. Tris eluate	0.060	129.0	0.011	39.6	2.64	29.2
Ammonium sulfate pptn. NaCl eluate	0.043	92.5	0.059	211.6	14.11	
Ammonium sulfate pptn. NaCl eluate	0.042	91.4	0.051	182.8	12.19	143.9

1. Volumes used in the assays: Initial supernatant, DEAE-eluents were 1.0 ml.
   Ammonium sulfate precipitation of eluates were 0.1 ml.

2. Buffers: 0.8 M Tris-HCl, 0.0045 M $CaCl_2$ (pH 8.0).
   0.8 M NaCl, 0.1 M Tris-HCl, 0.0045 M $CaCl_2$ (pH 8.0).

Appendix C.2.--Ammonium sulfate precipitation of the extracellular protease produced by Pseudomonas perolens ATCC 10757 from the DEAE-Sephadex A-50 eluate.

Treatment	Protein		Enzyme Activity			Spec. Act.
	O.D. 660 nm	mg Protein / ml	O.D. 660 nm	µg Tyr. / ml	Units	Units Enz. / mg Protein
Initial	0.244	0.19	0.063	6.12	1.63	9.0
Initial	0.243	0.19	0.063	6.12	1.63	9.0
30% Sat. supernatant	0.075	0.29	0.036	3.50	0.93	3.4
30% Sat. supernatant	0.071	0.28	0.029	2.82	0.75	2.7
30% Sat. pellet	0.086	0.34	0.036	3.50	0.93	2.9
30% Sat. pellet	0.081	0.32	0.035	3.40	0.91	2.9
50% Sat. supernatant	0.034	0.13	0.005	0.49	1.31	9.6
50% Sat. supernatant	0.041	0.16	0.005	0.49	1.31	9.6
50% Sat. pellet	0.069	0.05	0.314	30.49	8.10	188.5
50% Sat. pellet	0.068	0.05	0.224	21.75	5.80	134.5
70% Sat. supernatant	0.029	0.114	0.007	0.68	1.81	17.6
70% Sat. supernatant	0.030	0.118	0.002	0.19	0.51	5.0
70% Sat. pellet	0.050	0.04	0.028	2.72	0.73	25.5
70% Sat. pellet	0.000	0.00	0.002	0.19	0.05	0.0

Volume of assay: Initial (1.0 ml), other treatments (0.1 ml).

Appendix C.3.1.--The inhibition of Pseudomonas perolens protease by EDTA.

System	660 nm	µg Tyr.rel. / ml	Units	Units Enzyme / mg Protein	Inhibition
Initial	0.460	1,660	110.7		
Initial	0.436	1,572	104.8	651.0	--
1 mmole EDTA	0.427	1,541.2	102.8		
1 mmole EDTA	0.428	1,544.8	103.0	621.6	4.0
5 mmole EDTA	0.292	1,054.0	70.3		
5 mmole EDTA	0.298	1,075.6	71.7	428.9	34.0
10 mmole EDTA	0.029	104.8	7.0		
10 mmole EDTA	0.020	72.0	4.8	35.7	94.5
20 mmole EDTA	0.008	28.8	1.9		
20 mole EDTA	0.009	32.4	2.2	12.4	98.1

System: 0.5 ml EDTA (1,5,10 or 20 mmoles/tube).
0.5 ml Enzyme (0.166 mg or 108 enzyme units) in 0.1 M Tris HCl (pH 7.5).

Appendix C.3.2.--The effect of $CaCl_2$ addition to Pseudomonas perolens protease inhibited by 25 mmoles EDTA has upon activity.

$CaCl_2$ mmole	O.D. 660 nm	µg Tyr. / ml	Units	Enz. Units / mg Protein	$CaCl_2$ mmole	O.D. 660 nm	Tyr. / ml	Units	µg Tyr. / mg Protein
0	0.006	21.7	1.4		25	0.026	119.6	6.1	
	0.005	18.1	1.2	7.3		0.026	119.6	6.1	36.7
5	0.002	9.2	0.6		30	0.029	115.5	7.7	
	0.005	23.2	1.6	5.0		0.028	109.5	7.3	45.2
10	0.008	30.8	2.1		50	0.035	130.6	8.7	
	0.021	81.2	5.4	29.0		0.033	126.1	8.4	52.1
15	0.023	87.6	5.8						
	0.018	82.8	5.5	35.3					

System: 0.5 ml Enzyme (see Appendix 3.3.1).
0.25 ml (25 mmoles EDTA).
0.25 ml (50, 30, 25, 15, 10 and 5 mmoles $CaCl_2$).

Appendix C.3.3.--The ability of Pseudomonas perolens ATCC 10757 protease to hydrolyse N-CBZ-glycyl-L-leucine.

mM	Time (sec)	O.D. 570 nm	µM Leucine µl ml	µM Leucine ml min.
2	0	0	0	--
	15	0.019	13.1	--
	30	0.045	31.0	--
	45	0.080	55.3	84.4
4	0	0	0	--
	15	0.029	20.1	--
	30	0.068	47.3	--
	45	0.102	71.0	101.8
6	0	0	0	--
	15	0.039	27.1	--
	30	0.068	58.7	--
	45	0.099	86.1	118.0
8	0	0	0	--
	15	0.057	39.3	--
	30	0.118	82.1	--
	45	0.246	170.9	131.6

Appendix D.1.1. Animal live weights at the time of bleeding.

Treatment	Rabbit No.	Weight, gms					
		I	2	3	4	5	F
Fed	1	3,103	3,242	3,312	3,461	3,417	3,482
	5	3,283	3,319	3,353	3,385	3,389	3,517
	13	3,188	3,227	3,240	3,328	3,325	3,434
	19	3,198	3,303	3,400	3,466	3,388	3,468
$\bar{x}$		3,213	3,272.8	3,326.3	3,410	3,379.8	3,475.3
s		47.1	45.1	67.8	66.1	38.9	34.3
Fasted	3	3,195	2,882	2,669	2,497	2,367	2,253
	7	3,412	3,116	2,678	2,656	2,522	2,123
	8	3,326	2,917	2,675	2,539	2,347	2,266
	11	3,383	2,918	2,628	2,414	2,378	2,177
	15	3,052	3,755	2,629	2,220	2,177	1,773
	16	3,493	3,002	2,621	2,201	--	--
	23	3,149	2,782	2,632	2,352	2,215	1,753
	24	3,352	3,207	2,942	2,690	2,499	2,326
$\bar{x}$		3,295.4	2,947.4	2,684.3	2,434.9	2,357.9	2,095.9
s		148.9	155.5	106.7	168.6	129.5	236.5

Appendix D.1.2.--Total and individual muscle and organ weights.

Rabbit No.	Muscle Weights, gms			Organ Weights, gms	
	Total	Semitend.	Longis.	Liver	Heart
11	278.9	11.1	50.0	29.34	4.81
24	324.6	12.8	61.0	33.96	5.75
8	304.3	11.6	56.9	27.80	5.44
1	485.8	20.8	108.4	84.64	9.02
19	453.9	20.7	101.3	79.93	6.90
23	191.6	8.6	33.1	22.58	3.92
15	146.8	9.7	30.9	21.60	3.80
5	528.2	16.9	107.1	73.28	6.16
13	487.7	15.4	96.6	70.66	6.47
7	275.8	10.7	51.9	29.94	4.64
3	241.8	9.5	46.3	29.48	4.81
Fed					
$\bar{x}$	488.9	18.5	103.4	77.1	6.64
s	30.4	2.7	5.5	6.4	0.40
Fasted					
$\bar{x}$	252.0	10.6	47.2	27.8	4.70
s	63.4	1.4	11.4	4.4	0.66

Appendix D.2.1.--Blood glucose levels (mg%/ml) of fed and fasted (28 days) rabbits.

Treatment	Rabbit No.	Glucose (mg%/ml) at Bleeding					
		I	2	3	4	5	F
Fed	1	127.6	128.6	133.7	121.0	116.3	121.9
	5	--	122.8	111.7	114.7	119.1	111.1
	13	117.6	124.8	107.7	112.7	--	118.8
	19	129.3	125.9	122.3	129.7	135.6	145.1
$\bar{x}$		124.8	125.5	118.9	119.5	122.0	124.1
s		6.3	2.4	11.7	7.7	7.6	14.7
Fasted	3	150.0	107.2	--	96.0	116.7	117.0
	7	124.1	110.7	124.3	95.0	126.0	133.0
	8	107.6	111.7	143.7	100.7	--	132.3
	11	114.5	115.9	106.3	104.7	114.2	153.5
	15	130.0	107.6	117.7	107.0	115.3	102.4
	16	151.7	113.4	135.2	107.3	117.4	148.3
	23	130.0	105.5	101.7	96.7	101.4	134.4
	24	124.8	109.3	109.0	--	107.3	256.6
$\bar{x}$		129.1	110.2	119.8	101.2	115.4	121.4
s		15.4	3.5	15.6	4.9	8.9	15.1

Appendix D.2.2.--Total free amino acids in serum of fed and (28 day) fasted rabbits.

Treatment	Rabbit No.	Amino Acids (μM citrulline/ml) at Bleeding					
		I	2	3	4	5	F
Fed	1	5.06	6.07	6.32	4.76	--	5.28
	5	--	6.40	6.63	4.76	5.66	5.45
	13	4.60	6.07	5.36	4.12	5.92	5.02
	19	4.30	3.91	4.79	4.46	4.18	4.34
$\bar{x}$		4.65	5.61	5.77	4.52	5.25	5.02
s		0.39	1.15	0.85	0.30	0.94	0.49
Fasted	3	4.44	5.13	--	4.38	4.17	4.41
	7	5.13	5.89	5.26	3.81	5.32	5.03
	8	5.99	6.45	5.73	4.61	--	5.02
	11	4.92	4.93	3.78	4.07	4.50	5.22
	15	5.38	5.13	5.47	4.80	4.65	7.77
	16	4.48	5.43	7.01	4.80	4.55	4.68
	23	5.60	4.21	4.03	4.59	3.75	8.19
	24	5.15	5.42	5.87	4.92	5.46	4.77
$\bar{x}$		5.13	5.32	5.45	4.50	4.63	5.64
s		0.53	0.66	0.97	0.39	0.60	1.47

Appendix D.2.3.--Nonesterified free fatty acids in the serum of fed and (28 day) fasted rabbits.

Treatment	Rabbit No.	Nonesterified Free Fatty Acids, nmoles/ml					
		I	2	3	4	5	F
Fed	1	131.0	136.5	129.5	187.5	180.0	104.5
	5	--	101.0	89.5	260.0	135.5	130.5
	13	136.5	181.0	176.5	--	--	187.0
	19	124.5	--	125.5	167.0	98.0	203.0
$\bar{x}$		130.7	139.5	130.3	204.8	137.8	156.3
s		6.0	40.1	35.7	48.9	41.1	46.5
Fasted	3	118.5	314.0	--	333.0	295.0	407.0
	7	100.0	333.5	273.0	260.0	355.0	192.5
	8	134.0	397.0	277.5	408.5	--	--
	11	121.5	331.5	--	346.5	300.0	276.5
	15	133.5	253.0	343.0	262.0	314.5	49.5
	16	158.0	185.5	172.5	269.5	332.5	--
	23	104.0	364.5	335.5	273.0	310.5	63.5
	24	69.0	248.5	222.0	230.5	--	263.5
$\bar{x}$		117.3	290.9	270.6	297.9	317.9	207.3
s		26.3	86.7	65.6	59.2	22.4	136.0

Appendix D.3.1.--Effect of fasting upon the individual components of skeletal muscle, Longissimus. Values in mg/g.

Treatment	Rabbit No.	Total Protein	Myofib. Protein	Sarcopl. Protein	Stroma Protein	NPN
Fed	1	231.9	117.4	54.5	25.0	34.9
	5	250.1	131.7	65.1	16.6	36.9
	13	242.3	99.8	43.0	60.3	39.2
	19	244.3	101.2	57.6	42.7	42.8
$\bar{x}$		242.2	112.5	53.4	36.2	38.5
s		9.2	15.1	7.6	19.4	3.4
Fasted	3	228.6	120.4	55.6	12.8	39.7
	7	199.9	120.6	67.5	--	37.2
	8	225.2	119.4	50.8	7.9	47.1
	11	263.8	66.2	44.9	119.0	33.7
	15	225.7	45.7	49.3	86.6	44.1
	23	212.3	88.1	63.6	9.1	51.5
	24	219.2	110.2	59.3	9.8	39.6
$\bar{x}$		225.0	95.8	55.9	40.9	41.8
s		19.8	30.1	8.1	49.1	6.1

Appendix D.3.2.--Effect of fasting upon the individual components of skeletal muscle, Semitendinosus. Values in mg/g.

Treatment	Rabbit No.	Total Protein	Myofib. Protein	Sarcopl. Protein	Stroma Protein	NPN
Fed	1	231.4	112.9	51.3	33.2	34.0
	5	226.5	108.1	51.0	40.7	26.8
	13	227.2	117.0	54.1	22.3	33.8
	19	236.8	124.4	60.4	17.5	34.5
$\bar{x}$		230.5	115.6	54.2	28.4	32.3
s		4.7	6.9	4.9	10.5	3.7
Fasted	3	227.8	109.6	44.0	40.6	33.6
	7	205.7	112.2	41.4	17.9	34.2
	8	219.0	108.2	41.8	42.4	26.6
	11	218.7	108.8	48.8	35.5	25.6
	15	206.4	87.2	44.5	36.1	38.6
	23	219.1	95.3	28.3	55.3	40.2
	24	220.5	109.7	45.2	31.4	34.4
$\bar{x}$		216.7	104.4	44.3	37.0	33.3
s		8.0	9.4	2.7	11.4	5.5

Appendix D.3.3.--Catheptic activity present in calcium activated sarcoplasmic factor and kinase activating factor preparations. (Adapted from Bodwell and Pearson, 1964.)

Preparation	Time (min)	O.D. 280 nm	Change O.D. 280 nm	Corrected O.D. 280 nm
CASF-Fed-Peak 1	0	0.265	0.011	0.009
CASF-Fed-Peak 1	30	0.276		
CASF-Fed-Peak 2	0	0.249	0.024	0.022
CASF-Fed-Peak 2	30	0.273		
CASF-Fast-Peak 1	0	0.240	0.036	0.034
CASF-Fast-Peak 1	30	0.276		
CASF-Fast-Peak 2	0	0.241	0.030	0.028
CASF-Fast-Peak 2	30	0.271		
CASF-Fast-Peak 3	0	0.289	0.000	0.000
CASF-Fast-Peak 3	30	0.289		
Crude Homogenate	0	0.280	0.011	0.009
Crude Homogenate	30	0.291		
Blank	0	0.292	0.002	0.000
Blank	30	0.294		

1. Total reaction mixture volume: 2.0 ml.

2. Reaction mixture diluted to 10 ml before reading.

Appendix E.1.1.--Comparative proteolytic activities for proteases of different tissue origin or isolation.

Protease	O.D. 660 nm	µg Tyr. Rel. / ml	Enz.U. / Minute	Addition of 5 mM EGTA		
				O.D. 660 nm	µg Tyr.Rel. / ml	Enz.U. / Minute
CASF-Fed	0.027	228.6	15.2	0.003	28.6	1.9
CASF-Fed	0.026	247.6	16.5	0.001	9.5	0.6
CASF-Fasted	0.106	1,009.6	67.3	0.006	57.1	3.8
CASF-Fasted	0.106	1,009.6	67.3	0.007	66.7	4.4
KAF	0.024	228.6	15.2	0.009	85.7	5.7
KAF	0.022	209.5	14.0	0.007	66.7	4.4
Ps. perolens	0.146	6,952.4	456.8	0.044	419.1	27.9
Ps. perolens	0.159	7,571.4	504.8	0.045	428.6	28.6

1. Enzyme dilutions: Ps. perolens 1:10, all others 1:2.
2. Enzyme concentrations:

CASF-Fed	24 µg/ml
CASF-Fast	98 µg/ml
KAF	74 µg/ml
Ps. perolens	11 µg/ml

Appendix E.1.2.--Phosphorylase kinase activating activity.

Treatment	O.D. 380 nm	µg Phosphate / Test Tube	µmole Phos. / ml	KAF Units
CASF-Fed	0.165	4.37	9.79	4.90
CASF-Fed	0.167	4.42	9.90	4.95
CASF-Fast	0.208	5.50	12.32	6.16
CASF-Fast	0.213	5.63	12.61	6.30
KAF	0.058	1.53	3.42	1.71
KAF	0.065	1.72	3.85	1.97
Ps. perolens	0.064	1.69	3.78	1.89
Ps. perolens	0.070	1.85	4.15	2.08

Appendix E.1.3.--The effect of bovine heart KAF inhibitor upon the activity of the various proteases.

Protease	Inhibitor	O.D. 660 nm	µg Tyrosine / ml	Enzyme Units / Minutes
CASF-Fed	-	0.021	100.0	6.7
CASF-Fed	-	0.023	109.5	7.3
CASF-Fed	+	0.000	0.0	0.0
CASF-Fed	+	0.001	9.6	0.6
CASF-Fasted	-	0.098	466.7	31.1
CASF-Fasted	-	0.100	476.2	31.8
CASF-Fasted	+	0.007	66.6	4.4
CASF-Fasted	+	0.000	0.0	0.0
KAF	-	0.019	90.5	6.0
KAF	-	0.023	109.5	7.3
KAF	+	0.000	0.0	0.0
KAF	+	0.000	0.0	0.0
Ps. perolens	-	0.163	7,761.9	517.5
Ps. perolens	-	0.171	8,142.9	542.9
Ps. perolens	+	0.169	8,048.0	536.5
Ps. perolens	+	0.152	7,240.0	482.7

1. Enzyme dilutions: Ps. perolens 1:10, all others 1:2.

2. Enzyme concentration:
   - CASF-Fed 26 µg/ml
   - CASF-Fasted 94 µg/ml
   - KAF 74 µg/ml
   - Ps. perolens 12.1 µg/ml

3. Inhibitor concentration: 137.1 µg/ml

Appendix F.1.1.--Myofibrillar degradation study.

Protease	EDTA 10 mM	$CaCl_2$ 10 mM	Enzyme Concentration (µg/ml)
CASF-Fed	+		9.6
CASF-Fed		+	9.6
CASF-Fasted	+		9.8
CASF-Fasted		+	9.8
KAF	+		9.8
KAF		+	9.8
Ps. perolens	+		9.8
Ps. perolens		+	9.8
Control	+		0.0
Control		+	0.0

Basic system:

0.4 ml	100 mM KCl 20 mM Tris-Acetate (pH 7.0)
0.1 ml	10 mM $CaCl_2$ or EDTA
0.1 ml	50 mg/ml myofibrils
0.4 ml	Enzyme + buffer

Appendix F.1.2.--Molecular weight determination with SDS polyacrylamide gel electrophoresis.

Protein	Molecular Weight	Relative Mobility
Trypsin	21,000	0.88
Ovalbumin	43,000	0.60
Bovine serum albumin	68,000	0.41
Phosphorylase b	94,000	0.31
Kinase Activating Factor	--	0.24
Kinase Activating Factor	--	0.77
Calcium Activated Sarcoplasmic Factor	--	0.21
Calcium Activated Sarcoplasmic Factor	--	0.75
Ps. perolens protease	--	0.59