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A STUDY OF THE ACTION OF BOVINE CATHEPSIN D ON
INTRAMUSCULAR CONNECTIVE TISSUE

by

Elimosaria Elikalia Maeda

A dissertation submitted in partial fulfillment
of the requirements for the degree

of

DOCTOR OF PHILOSOPHY

in

Nutrition and Food Sciences

Approved:

UTAH STATE UNIVERSITY
Logan, Utah

1983

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Elimosaria E. Maeda.

DEDICATION

I am dedicating this work to my Dad Elikalia Mariti Maeda and Mom Elizabeth Mashina for the love and care they had for me during my childhood, and the very inspiration they imparted on me to develop interest in knowledge.

TABLE OF CONTENTS

| | Page |
|--|------|
| ACKNOWLEDGEMENTS..... | ii |
| DEDICATION..... | iii |
| TABLE OF CONTENTS..... | iv |
| LIST OF TABLES..... | vi |
| LIST OF FIGURES..... | vii |
| ABSTRACT..... | ix |
| INTRODUCTION..... | 1 |
| LITERATURE REVIEW..... | 5 |
| The Cathepsins..... | 5 |
| Cathepsin D Mode of Action on Natural and Synthetic Substrates..... | 14 |
| Multiplicity of Cathepsin D and the Associated Implications..... | 18 |
| The Nature of the Active Site on the Cathepsin D Molecule..... | 23 |
| The Action of Cathepsin D on Bovine Hemoglobin (The most Commonly Used Substrate for Cathepsin D Assay)..... | 25 |
| Cathepsin D Inhibition..... | 27 |
| Purification of Cathepsin D by Affinity Chromatography..... | 32 |
| Studies Implicating Cathepsin D in Connective Tissue Breakdown..... | 36 |
| The Role of Cathepsin D in Meat Tenderization..... | 39 |
| MATERIALS AND METHODS..... | 43 |
| Cathepsin D Extraction from Bovine Sternomandibularis Muscle..... | 43 |
| Extraction of Cathepsin D from Bovine Spleen..... | 45 |
| Purification of Cathepsin D by Affinity Chromatography..... | 45 |

Cont.

Table of contents Cont.

| | Page |
|--|------|
| Purification of Muscle Cathepsin D by Ion Exchange Chromatography and Gel Filtration Chromatography..... | 49 |
| Cathepsin D Activity Assays..... | 50 |
| SDS-Gel Electrophoresis of the pH 8.6 Eluate..... | 55 |
| Studies on Connective Tissue..... | 58 |
| RESULTS AND DISCUSSION | 65 |
| General Observations on Procedures for Purification of Bovine Cathepsin D | 65 |
| Characterization of Bovine Cathepsin D..... | 81 |
| Nature of Isolated Intramuscular Connective Tissue | 98 |
| Action of Cathepsin D and Bromelain on Intramuscular Connective Tissue..... | 101 |
| CONCLUSION..... | 111 |
| LIST OF REFERENCES..... | 113 |
| APPENDICES..... | 125 |
| Appendix A..... | 126 |
| Appendix B..... | 128 |
| Appendix C..... | 129 |
| Appendix D..... | 134 |
| Appendix E..... | 136 |
| VITA | 137 |

LIST OF TABLES

| Table | Page |
|--|------|
| 1. Determination of glass bead-pepstatin affinity column capacity for bovine spleen cathepsin D | 73 |
| 2. Purification table for bovine spleen cathepsin D by affinity chromatography using a glass bead pepstatin column | 77 |
| 3. Purification of bovine muscle cathepsin D by affinity chromatography | 87 |
| 4. Purification of bovine muscle cathepsin D by ion exchange chromatography using DEAE-Sephadex A-50 followed by gel filtration using Sephadex G-100 | 94 |
| 5. Percentage collagen in intramuscular connective tissue (IMCT) isolated from beef shank muscle | 99 |
| 6. Preparation of L-tyrosine standard curve | 126 |
| 7. The relative mobility of low molecular weight protein standard proteins in 7.5 and 10 % sodium dodecyl sulfate polyacrylamide gels..... | 129 |
| 8. The relative mobilities of high molecular weight protein standards after SDS-polyacrylamide gel electrophoresis using 7.5 % gels at pH 8.0 | 132 |
| 9. Solubilization of collagen in intramuscular connective tissue by pineapple stem bromelain | 134 |
| 10. A summary of the major features which distinguish the cathepsins | 136 |

LIST OF FIGURES

| Figure | Page |
|---|------|
| 1. Protein as measured by absorbance at 280 nm on bovine muscle fractions eluted from a glass bead-pepstatin affinity column. | 67 |
| 2. Protein as measured by absorbance at 280 nm on bovine spleen fractions eluted from a glass bead-pepstatin column | 69 |
| 3. The effect of pH on bovine muscle cathepsin D proteolytic activity..... | 75 |
| 4. Effect of pH on bovine spleen cathepsin D activity | 76 |
| 5. Effect of incubation time on the proteolytic activity of bovine spleen cathepsin D at pH 3.2. | 80 |
| 6. SDS gel-electrophoretic pattern of bovine spleen cathepsin D in 10 % gels at pH 8.6 | 82 |
| 7. SDS-gel electrophoretic pattern of a commercial preparation of bovine spleen cathepsin D | 84 |
| 8. SDS-polyacrylamide gel-electrophoresis of bovine muscle cathepsin D purified on a glass bead-pepstatin column | 88 |
| 9. A schematic presentation of the probable mechanism by which native bovine muscle cathepsin D undergoes autodegradation | 90 |
| 10. Elution pattern of bovine muscle cathepsin D on DEAE-Sephadex A-50 (1.0 X 50 cm column) | 92 |
| 11. Elution pattern of concentrated DEAE-Sephadex A-50 (fractions from bovine muscle) on a Sephadex G-100 column (1.0 X 25 cm column) | 93 |

Cont.

List of Figures Cont.

| | Page |
|--|------|
| 12. SDS-polyacrylamide gel electrophoresis of bovine muscle cathepsin D fractions eluted from DEAE-Sephadex A-50, and further purified by gel filtration on Sephadex G-100 | 95 |
| 13. SDS-polyacrylamide gel electrophoresis of intramuscular connective tissue in 7.5 % gels | 100 |
| 14. Effect of incubation time and pH on the extent of release of soluble connective tissue incubated with pineapple stem bromelain | 103 |
| 15. Electrophoresis of intramuscular connective tissue digest filtrates following incubation with pineapple stem bromelain at pH 3.0. | 105 |
| 16. L-tyrosine standard curve | 127 |
| 17. Protein standard curve | 128 |
| 18. Standard plot of molecular weight weight vs mobility for low molecular weight protein standards in 10 % polyacrylamide gels | 130 |
| 19. Standard plot of molecular weight vs mobility for low molecular weight protein standards in 7.5 % gels | 131 |
| 20. Standard plot of molecular weight vs mobility for high molecular weight protein standards in 7.5 % gels | 133 |
| 21. Hydroxyproline standard curve..... | 135 |

ABSTRACT

A Study of the Action of Bovine Cathepsin D on
Intramuscular Connective Tissue

by

Elimosaria E. Maeda, Doctor of Philosophy
Utah State University, 1983

Major Professor: D. P. Cornforth

Department: Nutrition and Food Science

Cathepsin D has been postulated to play a role in the tenderization of meat with ageing. Meat toughness has been related to connective tissue content and degree of myofibrillar shortening. This investigation was undertaken to determine the activity of cathepsin D on bovine intramuscular connective tissue.

Highly purified cathepsin D preparations from bovine spleen and bovine sternomandibularis muscle were obtained by ammonium sulfate fractionation followed by affinity chromatography on pepstatin coupled to controlled pore size alkyl amine glass beads. Maximum proteolytic activity using hemoglobin as substrate was obtained at pH 3.5 and 3.8 for bovine spleen and muscle cathepsin D, respectively. The banding pattern of bovine spleen cathepsin D on SDS-polyacrylamide gels was identical to that of a commercial preparation. Both cathepsin D preparations had 2 major

proteins with molecular weights of about 45,000 and 35,000 daltons. Bovine muscle cathepsin D preparations had 4 polypeptide bands corresponding to molecular weights of 57,000, 45,000, 41,000, and 20,000 on SDS gels. The 45,000 and 41,000 dalton components of muscle cathepsin D were shown to undergo degradation to release peptides of about 20,000 daltons during refrigerated storage of the 40-70 % ammonium sulfate fraction.

Cathepsin D from neither of the tissues had proteolytic action towards intramuscular connective tissue isolated from bovine shank muscle over the pH range 3.0-4.5 following incubation for 24 h at 37 C. However, a commercial preparation of pineapple stem bromelain was found to degrade connective tissue, resulting in a release of 44,000 and 64,000 dalton peptides. Degradation was maximum at pH 3.0 and the rate of degradation decreased with pH increase. The failure of a highly purified cathepsin D preparation to degrade connective tissue is a clear indication that the meat tenderizing effect of cathepsin D during meat ageing is not due to its proteolytic action on intramuscular collagen, but due to its reported proteolytic action towards myofibrillar proteins.

(147 pages)

INTRODUCTION

Collagen, the principal component of connective tissue has been investigated with regard to its relationship to meat tenderness, but the results have been conflicting. Some workers have related total collagen to texture (Kim et al., 1967, and Field et al., 1969) while others have found little influence of collagen on tenderness (Herring et al., 1967, and Davey and Gilbert, 1968a).

Recently, Kopp and Valin (1981) reported that the muscle lysosomal fraction contains an enzyme system capable of reducing the strength of muscle collagen at pH 5.5. Cathepsins represent one group of the lysosomal enzymes and have been isolated from a variety of mammalian, avian and marine animal tissues. It is currently understood that the cathepsins are lysosomal in origin, and display activity under acid conditions. Food scientists are interested in the possible role played by the cathepsins in bringing about desirable changes to the muscle postmortem, especially if such changes involve tenderization. Additional interest in the cathepsins has been accumulating due to need for explanation of such observed phenomenon as resolution of rigor. There is evidence that cathepsin D has a degradative action on myofibrillar proteins (Schwartz and Bird, 1977; Robbins et al., 1979; Fan, 1981 and Makinodan et al., 1982). According to Robbins et al. (1979) cathepsin D digests Z-disk

proteins and myosin. Digestion of the Z-disk proteins is disruptive to the actomyosin anchorage which explains the observed resolution of rigor.

Thus far there is no convincing evidence that cathepsin D specifically attacks muscle connective tissue, a tissue that provides background toughness in meat. According to Burleigh et al. (1974) acid soluble fibrils and collagen fibrils were digested by cathepsin B1 but not cathepsin D. There have been reports which implicate cathepsin D in connective tissue degradation and particularly cathepsin D extracted from specialized organs like liver, spleen and uterus. Cathepsin D activity was reported to be pronounced in involuting rat uterus with such increase being associated with elevated levels in plasma and urinary free hydroxyproline (Woessner, 1962). According to Woessner (1962) the optimal pH for the activity of the enzyme in solubilization of uterine connective tissue was pH 3.5 which is also one of the reported optimum pH's for cathepsin D activity using bovine hemoglobin as substrate. According to Robbins and Cohen (1976) there was a structural alteration of bovine connective tissue on being incubated with spleen cathepsin D. Kruggel and Field (1971) observed a decrease in the quantity of crosslinks in intramuscular collagen during muscle aging. A similar observation was made by Ledward et al. (1975) who noted an increase in the amount of tendon collagen melting at less than 67 C in 7 out of 16 tendons previously stored at 1 C for two weeks. Recently Kopp and

Valin (1981) incubated collagen fibers with lysosomal enzymes and observed a significant decrease in fiber thermal stability.

It was on the basis of such studies that cathepsin D has been perceived as having a possible role in connective tissue degradation. There have also been suggestions to use bovine spleen cathepsin D as an exogenous meat tenderizer (Robbins et al., 1979). Connective tissue thermal stability changes (Kopp and Valin, 1981) or structural alterations as seen under the microscope (Robbins and Cohen, 1976) following incubation of connective tissue with a lysosomal enzyme may not be the most reliable criteria for evaluating connective tissue breakdown. Presence of low molecular weight peptides bearing hydroxyproline or free hydroxyproline in such digests may be a more reliable means of establishing whether connective tissue has undergone digestion.

The richest sources of cathepsin D in bovine tissue are spleen, liver and uterus (Venugopal and Bailey, 1978). The activity of the enzyme as isolated from these specialized organs is relatively high. Cathepsin D has also been isolated from chicken muscle (Iodice et al., 1966; Caldwell and Crosjean, 1971), rabbit muscle (Schwartz and Bird, 1977) and bovine muscle (Fan, 1981). The low activity of cathepsin D isolated from muscular tissue has been attributed to a possible adherence to myofibrillar proteins during homogenization procedures (Okitani et al., 1981). Problems in purification of cathepsin D and subsequent cha-

racterization have been encountered, and the explanations given so far relate to its instability to acid conditions (Smith and Turk, 1974) which are also conditions for its optimal enzymatic activity. The exact molecular characterization of muscle cathepsin D is still a debatable issue due to the possibility of autodegradation during purification. According to Okitani et al. (1981) the nature of muscle cathepsin D as characterized in vitro may not reflect its nature and possibly its action in vivo. Its possible role in connective tissue degradation during muscle aging has yet to be established.

It is the purpose of the present study to purify cathepsin D from bovine spleen and bovine muscle and use the purified enzyme in incubation studies with connective tissue extract from bovine shank muscle. The activity of cathepsin D towards purified intramuscular connective tissue as substrate will be characterized by sodium dodecyl sulfate (SDS) polyacrylamide gel electrophoresis. The digestive action of extracts from muscle and spleen will be compared with that of commercially available preparations of bovine spleen cathepsin D, and pineapple stem bromelain.

LITERATURE REVIEW

The Cathepsins

Cathepsins were partially purified for the first time by Anson (1938). His procedures have remained the basis for all cathepsin activity assays with slight modifications. According to Gianetto and de Duve (1955) difficulties have been encountered on adopting the original procedures of Anson (1938). Urea-denatured rather than acid denatured bovine hemoglobin has been reported to be a better substrate for activity assay (Sliwinski et al., 1959; Venugopal and Bailey, 1978). The term "cathepsin" has since been restricted to those acid proteases with proteolytic action on hemoglobin. Several distinct cathepsin types were isolated from bovine spleen by Press et al. (1960). Distinction of the types was based on the ability to digest specific synthetic peptides. The specific synthetic peptides used by Press et al. (1960) for distinguishing cathepsins A, B, and C were benzyloxycarbonyl-L-glutamyl-L-tyrosine, benzoyl-L-arginine amide, and a mixture of glycyl-L-tyrosine amide and hydroxylamine, respectively. The type of cathepsin that had no action on the synthetic substrates for cathepsins A, B, and C was named cathepsin D (Press et al., 1960). Over the past few years there has been isolation and characterization of new types of cathepsins from a variety of tissues and the types have assumed names like cathepsin E, L, H and S.

Cathepsins D and E are similar in that they are both inhibited by pepstatin. However, they differ in their molecular weight, substrate preference, pH for optimal activity, relative abundance in different tissues, heat sensitivity and relative electrophoretic mobility. Cathepsin E was isolated for the first time from rabbit bone marrow by Lapresle and Webb (1962). Cathepsin E was found incapable of hydrolysing the synthetic substrates for cathepsin A, B, and C. The cathepsin was also shown to occur in trace amounts in other tissues including the spleen and liver, both of which are known to be rich in cathepsin D. Unlike cathepsin D which prefers bovine hemoglobin as substrate, cathepsin E preferably digests bovine or human serum albumin at an optimal pH of 2.5 (Lebez et al., 1968; Yago and Bowers, 1975; Venugopal and Bailey, 1978; Lapresle and Webb, 1962; Cochrane and Aikin, 1966). However, cathepsins D and E digest hemoglobin as substrate at the same optimal pH (Turk et al., 1968). Turk et al. (1968) purified cathepsin E from bovine spleen which was reported to have an apparent molecular weight of $305,000 \pm 40,000$. This molecular weight was high in comparison with recently reported molecular weights ranging from 90,000 to 100,000 (Yamamoto et al., 1978; Jasani et al., 1978). Cathepsin D occurs mainly in the liver and spleen and is capable of cleaving the β -chain of insulin. Besides the common feature of being inhibited by pepstatin, cathepsin D and E are non-reactive towards thiol-group reactive agents like iodoacetate and p-chloro-

mercuribenzoate (Yago and Bowers, 1975). Venugopal and Bailey (1978) separated cathepsin D from cathepsin E by differential heat treatment. Incubation of the enzyme mixture at 45 C for 30 min inactivated cathepsin E leaving cathepsin D intact. Rabbit bone marrow cathepsin E was also reported by Lapresle and Webb (1962) to be heat labile. These findings are however contrary to the reported heat stability of cathepsin E (Jasani, 1973; and Jasani et al., 1978).

Inhibition of a cathepsin preparation by iodoacetate or p-chloromercurylbenzoate is an indication that the cathepsin is thiol-group dependent, which is a common characteristic of cathepsins B and S. Cathepsin B in human liver occurs in two forms named cathepsins B1 and B2 with molecular weights of 25,000 and 50,000 respectively (Barrett, 1972). Cathepsin B1 has ability to degrade insoluble collagen (Burleigh et al., 1974).

Cathepsins L and H were first isolated from rat thoracic duct lymphocytes and rat liver lymphoid tissue (Yago and Bowers, 1975) and recently from bovine spleen (Robbins and Walker, 1982). The amount of cathepsins H and L in rabbit tissues, bovine spleen and human tonsils is however very low (Yago and Bowers, 1975). Cathepsins H and L also have characteristics similar to those of cathepsin D, notably the ability to digest bovine hemoglobin at an optimum pH close to 3.5, noninhibition by thiol-reactive inhibitors and inhibition by pepstatin. In a recent study

however, cathepsin L was shown to be inhibited by haptoglobin using azocasein as substrate, suggesting cathepsin L to be a thiol-dependent proteinase and therefore similar to cathepsin B (Pagano et al., 1982). However cathepsins H and L were insensitive to the antisera that is inhibitory to cathepsin D (Yago and Bowers, 1975). Cathepsin H and L have molecular weights of 95,000 and 45,000 respectively; hence the code letters H and L standing for heavy and light chain forms respectively. The L form was produced without loss of activity on incubation of the H enzyme with 2-mercaptoethanol at 37 C, and this led to speculation that cathepsin H is a dimer of cathepsin L monomers joined by disulfide linkages.

Cathepsin C is distinguished from cathepsins A and B on the basis of reactivity towards its specific substrate as reported by Press et al. (1960). Cathepsin C is a dipeptidyl amino peptidase which takes part in deamination reactions. Huang and Tappel (1972) isolated cathepsin C from beef spleen and rat liver and reported it to be activated by L-cysteine, dithiothreitol, chloride and bromide ions, but strongly inhibited by iodoacetic acid and acetamide. Activation by cysteine and related compounds and inhibition by acetamide or iodoacetamide, are main features peculiar to sulfhydryl group dependent enzymes of which cathepsin C is a member.

A few other types of cathepsins have been isolated and partially characterized in recent years. Cathepsin G and S

are the most recent types. Roughley and Barrett (1977) observed cathepsin G to have pronounced action on proteoglycans. Cathepsin S was isolated from bovine spleen by Turk and Kregar (1978) and was found to have features similar to those of cathepsin B and D. Like cathepsin B, cathepsin S is a thiol-group dependent proteinase with a molecular weight of 23,000. Cathepsin S resembles cathepsin D in its ability to digest hemoglobin at pH 3.5, and is inhibited by pepstatin. However, it interacts with thiol reactive inhibitors, a feature which is not common to cathepsin D. A summary of the major characteristics which distinguish the cathepsins is given in APPENDIX E.

Localization of Cathepsin D and other Acid Proteases

Gianetto and de Duve (1955) were the first to observe increased release of cathepsins and other acid proteinases from rat liver particles due to cell disrupting processes such as homogenization and thermal shock. This led to speculation that acid proteases (cathepsins, β -glucuronidase, and acid phosphatase) are retained by sac-like granules where they are prevented from contact with protein substrates. In a subsequent study by Romeo et al. (1966) the acid proteases were found to be associated with cytoplasmic particles, the lysosomes. The study involved activity assays of ultracentrifugation fractions. A variety of cell injuring treatments including osmotic shock, prolonged homogenization and

solubilization of cell membrane by the nonionic detergent Triton X-100 promoted release of the acid proteinases from the cytoplasmic particles. Romeo et al. (1966) thus speculated that solubilization was achieved by action of the detergent on membrane phospholipids. The influence of such membrane disruptive and solubilization agents was also reported by Stagni and de Bernard (1968) who in addition observed that beef skeletal lysosomes were more resistant to osmotic shock than rat skeletal muscle lysosomes.

The existence of lysosomal particles in skeletal muscle had not been confirmed by the mid sixties. Bird et al. (1968) observed a structure-linked latency in cathepsin D activity since treatment of muscle cells with Triton X-100 and physiological solubilizers like vitamin A alcohol led to an increase in activity recovered in extramitochondrial fractions. Despite such evidence, lysosomes were still believed as being non-existent in normal skeletal muscle and the occurrence of these particles in muscle as reported earlier, was believed to have origin from macrophages and connective tissues in muscle tissue. Existence of lysosomal particles in normal skeletal muscle was confirmed by Canonico and Bird (1970) after subjecting postnuclear liver and muscle supernatants to zonal fractionation. Starvation, vitamin E deficiency and denervation were noted for inducing ultrastructural alterations culminating in formation of large secondary lysosomes. According to Dean (1975) the lysosomal membrane acts as a barrier to passage of protons

and the intralysosomal pH remains at a lower level relative to the pH of the cytosol. The membrane is however permeable to weak bases such as ammonia, methylamine and chloroquin. Upon crossing the barrier they pick up protons causing an increase in pH which creates an unfavorable environment for catheptic enzyme activity (Kay, 1978).

Distribution of Cathepsin D in Living Tissues

Cathepsin D has been isolated from various tissues such as kidney, spleen, liver, brain and muscle. It's activity in muscle is minimal. Iodice et al. (1966) isolated a cathepsin from chicken breast muscle which had an optimal activity at pH 2.8 using bovine hemoglobin as substrate. The relative activity of the muscular cathepsin D was about 5 % of that observed for spleen cathepsin D. The skeletal muscle cathepsin D was also associated with cathepsin A. Chromatographic separation of cathepsin A from cathepsin D rendered cathepsin A inactive (Iodice, 1967). It was therefore suggested that the peptides produced after cathepsin D proteolytic action were the substrates for cathepsin A. The coexistence of these two cathepsins in the intestinal mucosa of pig was also demonstrated by Kregar et al. (1967).

Cathepsin D as a Strict Acid Protease

Cathepsin D activity has been assayed within the pH range 2.8 to 5.0. In the classical procedure developed by

Anson (1938) the optimal pH for assay was 3.5. In subsequent reports, the assaying conditions described by Anson have been adopted, with slight modifications which do not involve a change of substrate (Press et al., 1960; Woessner, 1962; Kregar et al., 1967; Smith and Turk, 1974; Jasani et al., 1978; and Turk et al., 1977). Optimal pH conditions lower or above 3.5 have also been reported by some other investigators. Iodice et al. (1966) reported pH 2.8 as being optimal for assay of cathepsin D isolated from chicken muscle and the enzyme also had a relatively high activity at pH 3.8. This biphasic nature of muscle cathepsin D has also been reported by Bird et al. (1968) and more recently by Doke et al. (1980) for cathepsin D purified from muscle of the fresh water fish Tilapia mossambicus.

The biphasic nature of cathepsin D isolated from muscular tissues has continued to be a paradox. A Hofstee plot of the data reported by Bird et al. (1968) gave a perfectly linear relationship and existence of two independent enzymes simultaneously acting on the substrate was ruled out.

Under extreme pH conditions, cathepsin D has no proteolytic action on hemoglobin as substrate. Brostoff et al. (1974) reported less than 1 % of the activity at pH 3.5 on incubating assay mixtures at pH 2.0 and 7.0. In a few studies, higher pH conditions have been selected, with the general idea of minimizing enzyme denaturation while under incubation at 37 C for periods less than 10 min (Anson, 1938) up to 2 h (Dingle et al., 1971; Venugopal and Bailey,

1978). The pH range above 3.5 in which successful assays can be conducted is however narrow. Assays of cathepsin D from porcine and bovine muscular tissue and leucocytes, using urea denatured hemoglobin as substrate (Venugopal and Bailey, 1978) and from rabbit skeletal muscle (Okitani et al., 1981) is reported to have been successfully done at pH 4.0. However Gianetto and de Duve (1955) reported a 75 % reduction in cathepsin D activity at pH 5.0 relative to the activity at pH 3.5.

By and large, the optimal pH conditions for assay depends on the substrate acted upon. According to Knight and Barrett (1976) there is yet no suitable substrate which can effectively substitute for hemoglobin in the course of assaying cathepsin D activity. In a few cases the activity of cathepsin D towards other proteins such as bovine serum albumin is reported to be negligible. Barrett (1970) observed that human and chicken cathepsin D had no action towards acid denatured albumin at pH 3.5 and 3.0 respectively.

Dorer et al. (1978) attempted to study the action of cathepsin D on synthetic and natural substrates for renin, notably tetradecapeptide renin substrate. It was shown that cathepsin D purified from hog spleen released angiotensin D from tetradecapeptide renin substrate at an optimal pH of 4.5 and there was no activity at pH 7.0 which is consistent with the general behavior of the enzyme towards its sub-

strates under neutral pH conditions.

Cathepsin D Mode of Action on Natural and
Synthetic Substrates

The action of cathepsin D on natural and synthetic protein substrates followed by release of peptides bearing aromatic amino acids like tyrosine has long been known. In fact this was the basis of Anson's procedure in which the tyrosine equivalents of TCA soluble peptides were determined by employing the Folin & Ciocalteu reaction (Woessner and Brewer, 1963; Smith and Turk, 1974; Dorer et al., 1978; Yamamoto et al., 1979; Robbins et al., 1979; Doke et al., 1980; and Okitani et al., 1981). In recent years the majority of activity assays have been based on absorbance data at 280 nm for the TCA soluble peptides following incubation with the enzyme (Sliwinski et al., 1959; Robbins and Cohen, 1976; Keilova and Tomasek, 1976; Venugopal and Bailey, 1978; Jasani et al., 1978; Huang et al., 1979; Chian et al., 1981; and Chian et al., 1982). What is common to both approaches is the realization that peptides bearing aromatic amino acids notably tyrosine, are released during the proteolysis. In only one case a fluorescamine method has been reported (Yago and Bowers, 1975).

The ability to cleave the oxidized β -chain of insulin has been reported to be a confirmatory test for cathepsin D, and this test has also served as a reliable way of distinguishing cathepsin D from the other cathepsins (Press et

al., 1960; Barrett, 1970; Woessner and Shamberger, 1971; Sapolsky and Woessner, 1972; and Woessner, 1973). The procedure involved is however complex and time consuming (Lebez et al., 1968).

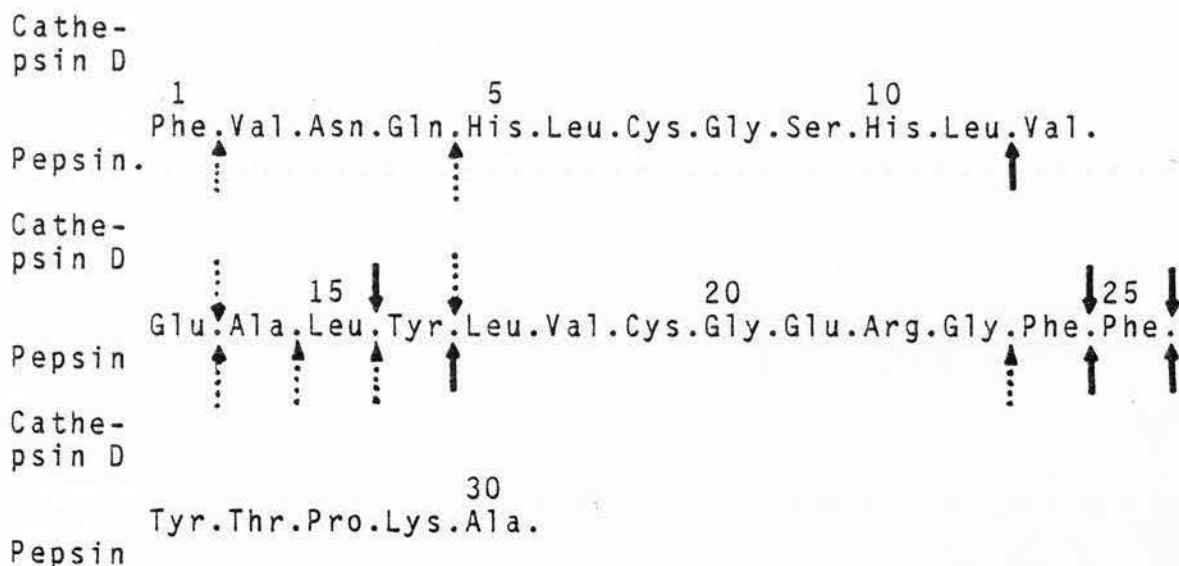
Amino acid sequence analysis coupled with peptide mapping of the released peptides have been the key to identification of the site(s) along the polypeptide chain cleaved by cathepsin D, and according to Woessner and Shamberger (1971) and Lin and William (1979) the sites are Leu-Phe; Phe-Phe; Phe-Tyr; Phe-Val; Ala-Ala and Ala-Tyr. In a previous study by Barrett (1970) it was shown that one or more of the cathepsin D enzyme forms from seven different tissue sources cleaved a total of 11 linkages on the oxidized β -chain of insulin. However, only three of the insulin bonds namely, Phe-Phe; Leu-Tyr; and Tyr-Leu were hydrolysed by all the seven forms of cathepsin D. In the same study it was shown that these bonds could be cleaved by pepsin. The characteristic feature common to all the cleaved bonds was the presence of aromatic residues. In a similar study by Kazakova and Orekhovich (1972) using synthetic heptapeptides as substrates the Phe-Phe bond was readily cleaved by cathepsin D enzyme prepared from bovine spleen, rat liver and ascite cells and this was in addition to other bonds containing nonpolar residues such as leucine. The similarity of pepsin and cathepsin D with respect to the nature of the bond cleaved has also been reported by Ferguson et al. (1973) who noted that the specificity of bovine spleen

cathepsin D and porcine pepsin A were identical in the course of cleaving a synthetic substrate bearing the group A-Phe(NO₂)-Phe-B (i.e Phe-Gly-His-Phe(NO₂)-Phe-Val-Leu-OMe). For both enzymes, hydrophobic residues formed a susceptible cleavage site.

Competitive inhibition studies using synthetic oligopeptides have in recent years provided an additional way of establishing the identity and nature of the bond susceptible to cathepsin D (and other acid proteinase) cleavage. Parikh and Cuatrecasas (1973) postulated that the site(s) of cleavage for the majority of acid proteinases like renin and pepsin is/are identical for all substrates. Substitution of a D-amino acid for a L-amino acid at the site of cleavage in the native renin substrate yielded competitive inhibitors. Lin and William (1979) observed that such substitution did not have to be at the cleavage site for cathepsin D to be competitively inhibited. Synthetic oligopeptides having a terminal carboxyl group and a D-amino acid situated third from the potentially cleavable bond (the Phe-Phe bond in peptide VI with the sequence L-Glu-D-Phe-Pro-Phe-Phe-Val-Trp) were effective competitive inhibitors of pepsin and cathepsin D.

The number of peptides released from specific proteins after cleavage by cathepsin D is variable and depends on the nature and number of susceptible bonds, the period of incubation and the source of the enzyme. Brostoff et al. (1974) showed that cathepsin D purified from bovine and

rabbit liver hydrolysed only the Phe-Phe bond of the A1 protein (Basic Protein) from bovine central nervous system leading to release of two peptides with residues 1-42 and 43-169. Resistance to cleavage of the Phe-Phe bond between residues 88-89 was attributed to either conformational restrictions or protection offered by the surrounding amino acids. Surprisingly however, both Phe-Phe bonds were readily cleaved by bovine brain cathepsin D leading to release of three peptides having residues 1-42, 43-88 and 89-169 (Brostoff et al., 1974). The cleavage sites on the β -chain of insulin by cathepsin D and pepsin are strikingly similar. Both enzymes attack at the carboxylic side of an aromatic amino acid or an amino acid with a longer side chain such as leucine (Keilova and Keil, 1968). The following cleavage scheme was proposed:-



The solid and dotted arrows indicate bonds that are strongly and weakly susceptible to cleavage, respectively. The most

susceptible sites are the regions between amino acid residues 11-16 and 24-26 (Keilova and Keil, 1968).

Chromatographic, electrophoretic, amino acid analysis and partial peptide sequencing studies enabled Whitaker and Seyer (1979b) to demonstrate that long incubation periods allow brain cathepsin D to further cleave the bovine Basic Protein (A1 protein) into a larger number of peptides in a sequential fashion which was characterized by a rapid initial cleavage of the Phe-Phe bond at residues 42 and 43 followed by the same type of bond at residues 88 and 89. New bonds susceptible to cathepsin D, but not reported in earlier studies, were also reported and these included Asn-Ile; Leu-Asp; and Phe-Lys. These findings ruled out a previously held generalization by Dingle et al. (1972) that the cleavage site had to have only aromatic or hydrophobic amino acid residues or bonds linking amino acids with hydrophobic side chains.

Multiplicity of Cathepsin D and The Associated Implications

Isolation of cathepsin D by different procedures has revealed the presence of multiple forms (isozymes) and to date it is still unclear whether multiplicity is confined in vivo to specific tissues or is a result of partial hydrolysis in the course of isolation. Multiplicity of cathepsin D has been reported on isolation of the enzyme from bovine spleen (Press et al., 1960; Fergusson et al., 1973), bovine

uterus, (Sapolsky and Woessner, 1972), rat brain (Marks and Lajtha, 1965), and chicken liver (Barrett, 1972).

Resolution of cathepsin D preparation into multiple forms has been possible by procedures involving rechromatographing of cathepsin D isolated at different pH values (Press et al., 1960); disc electrophoresis (Woessner and Shamberger, 1971) and isoelectric focussing (Barrett, 1970; Sapolsky and Woessner, 1972; Ferguson et al., 1973; Huang et al., 1979; Robbins and Walker, 1982). Robbins and Walker (1982) demonstrated that there are at least 7 isozymes of spleen cathepsin D having isoelectric points (pI) ranging from 5.25 to 6.80. These reports were in close agreement with the findings by Press et al. (1960), who reported the presence of at least 10 isozymes of cathepsin D from bovine spleen. The isoelectric points also fall within the range reported by other investigators. Smith and Turk (1974) isolated 3 isozymes from bovine spleen with pI values of 5.6, 5.9, and 6.4. Ferguson et al. (1973) reported pI values of 6.1, 6.3, and 6.7 while Huang et al. (1979) reported values of 6.49 and 6.04 for bovine spleen cathepsin D isozymes.

Despite the apparent existence of multiple forms of cathepsin D, there seems to be homogeneity with regard to molecular weight of some forms with the main differences being their individual isoelectric points and specific activities. The several distinct forms of cathepsin D isolated from bovine uterus had a molecular weight of 43,000 as determined by molecular sieve chromatography on Sephadex G-

100 and Sephadex G-75 (Woessner and Shamberger, 1971). Similar results were obtained by Barrett (1970) who reported three isozymes of chicken and human liver cathepsin D as having a molecular weight of 45,000 after resolving the enzyme by isoelectric focusing and independent molecular weight determinations by gel filtration on Sephadex G-100 and by SDS-polyacrylamide gel electrophoresis.

Several of the 12 isozymes (form 1 to 12) of bovine spleen cathepsin D isolated by Sapolsky and Woessner (1972) gave a single band on being resolved by SDS-polyacrylamide gel electrophoresis. Three isozymes (forms 2 to 4) gave a single band corresponding to molecular weight of 40,000 to 42,000. Forms 5 and 6 gave three distinct bands corresponding to molecular weights ranging from 13,000 to 14,000. Sapolsky and Woessner (1972) thus proposed that form 4 was the primary form comprised of a single polypeptide with a molecular weight of 40,000 which is cleaved into two fragments of two thirds and one third the length of the parent peptide without loss of activity. This theory was supported by the findings of Ferguson et al. (1973) who reported the appearance of a subunit having a molecular weight of 28,000 following the resolution of bovine spleen cathepsin D by SDS-polyacrylamide gel electrophoresis, whose molecular weight estimation by molecular sieve chromatography on Sephadex G-100 was 42,000.

Contrary to the observations that cathepsin D exists in multiple forms, Smith and Turk (1974) reported molecular

weight homogeneity for bovine spleen and thymus cathepsin D purified by affinity chromatography on a hemoglobin-Sepharose-4B column, followed by SDS-polyacrylamide gel electrophoresis. A single band corresponding to an apparent molecular weight of 42,000 was obtained. Smith and Turk (1974) therefore proposed affinity chromatography as a rapid procedure for purification of cathepsin D so as to minimize autodegradation. In a subsequent study by Kazakova and Orekhovich (1976) cathepsin D was rapidly isolated from chicken liver by affinity chromatography on AH-Sepharose 4B-pepstatin followed by assessment of enzyme homogeneity on SDS-polyacrylamide gel electrophoresis. A single band was observed. The results of cathepsin D purification by affinity chromatography appeared to suggest non-existence of multiplicity of the enzyme in vivo. Demonstration of such a phenomenon in earlier studies was a result of enzyme autolysis into active peptides during purification by exceedingly long procedures. Recently however, purification of porcine spleen cathepsin D by affinity chromatography on AH-Sepharose 4B-pepstatin still revealed the existence of at least 6 isozymes (Huang et al., 1979). Each of the isozymes, I to IV had a molecular weight of 50,000, being a composite of two subunits of molecular weights of 35,000 and 15,000 daltons. The fifth isozyme was a single polypeptide of molecular weight 100,000. Huang et al. (1979) speculated that the high molecular weight isozyme (MW=100,000) is the precursor form of a single chain species (MW=50,000) which

in turn becomes converted to two subunits having molecular weights of 35,000 and 15,000. Supposedly, these conversions were taking place in vivo (Huang et al., 1979).

Cathepsin D purification and characterization within the last decade tend to suggest that multiplicity is a feature common to cathepsin D preparations isolated from major organs such as spleen, liver and brain. On the contrary, cathepsin D multiplicity has not been demonstrated in muscle and closely related tissues. Cathepsin D isolated from the intestinal mucosa of pig had a molecular weight of 37,000 (Kregar et al., 1967). In a recent study by Doke et al. (1980) cathepsin D from the skeletal muscle of fresh water fish (Tilapia mossambicus) was found to have a molecular weight of 38,000 based on molecular sieve chromatography on Sephadex G-100. Independent molecular weight estimation of rabbit muscle cathepsin D by molecular exclusion chromatography and SDS-polyacrylamide gel electrophoresis showed the same molecular weight of 42,000 (Okitani et al., 1981). These results suggested that muscle cathepsin D has no subunit structure.

The Nature of the Active Site on the Cathepsin D Molecule

Recent studies have indicated a structural homology among the acid proteinases. X-ray diffraction studies at 3.0 Angstrom resolution on two fungal acid proteinases revealed identical secondary and tertiary structures (Subramanian et

al., 1977). The enzymes were bilobal and had a pronounced cleft between the two lobes which was identified as being the active site region after conducting binding studies with pepstatin.

The studies which led to characterization of pepsin about a decade ago, have in recent years been extended to cathepsin D, and the findings so far tend to confirm a probable homology among the acid proteinases. Similarity among the acid proteinases has been confirmed with respect to structure, catalytic mechanism, sequence and active site similarity, high preference for hydrophobic sites on their substrates, activity within the acid range, inhibition by synthetic and natural oligopeptides, and by epoxy and diazo compounds. All these features led Huang et al. (1979) to predict a common evolutionary origin of cathepsin D with gastric and other acid proteinases.

The identification and characterization of the active site in pepsin and other acid proteinases has been made possible by use of active site-directed reagents. Tang (1971) observed that pepsin was inhibited after treatment with epoxide and diazo compounds, both of which are active site directed reagents. Amino acid sequence analysis of modified pepsin revealed that inhibition was due to esterification of aspartyl residues (Chen and Tang, 1972). At about the same time, Hartsuck and Tang (1972) established that enzymatic activity was abolished after the modification of a single aspartyl carboxylate side chain in pepsin by

1,2-(p-nitrophenoxy) propane. The degree of inactivation was related to the incorporation of the modifier in a 1:1 molar ratio suggesting that only one aspartyl residue was affected. Similar findings have also been reported by Huang et al. (1979) and Lin and William (1979) who confirmed specific modification of Asp 32 and Asp 215 by epoxides and diazo inactivators respectively. Tang et al. (1973) had attributed the catalytic activity to Asp 32 and Asp 215 in the pepsin sequence.

Attempts have also been made to identify and partially characterize the active site in cathepsin D. Turk et al. (1977) have reported that bovine spleen cathepsin D was inhibited by diazo-acetyl-DL-norleucine methyl ester (a diazo compound) and epoxy-3-(p-nitrophenoxy) propane (an epoxide compound). Such inhibition was due to modification of aspartyl residues which are apparently essential for enzymatic activity. Cathepsin D isozymes isolated from porcine spleen have also been reported to be inactivated by epoxide and it was speculated that inhibition was through esterification of Asp 32 with residue 215 being a diazo reactive aspartate (Huang et al., 1979) which is in agreement with an earlier observation by Keilova and Tomasek (1976).

The aspartic acid residues can play a catalytic role, when in the un-ionized state. The un-ionized form facilitates formation of a tetrahedral intermediate (Knight and Barrett, 1976). This is in agreement with the high activity

displayed by these enzymes in the acid pH range. Complete loss of cathepsin D activity towards hemoglobin on going from pH 5.0 to 7.0 is probably due to abolition of the substrate binding site resulting from conformation change (Knight and Barrett, 1976).

The Action of Cathepsin D on Bovine
Hemoglobin (The most Commonly
Used Substrate for
Cathepsin D Assay)

Kinetic studies on interaction of cathepsin D with its substrate, bovine hemoglobin, was studied by Bird et al. (1968). An Eadie-Hofstee plot of the kinetic data was linear indicating that hemoglobin was forming a complex that remained at a relatively constant concentration throughout the period of observation at 37 C, pH 3.9. A K_m (Michaelis constant) of 0.08 % w/v hemoglobin was obtained on plotting the data by either the Eadie-Hofstee or a modified Lineweaver-Burk method. (Note: The constant K_m is not usually expressed in % units but in molar units). In an earlier study by Sapolsky and Woessner (1972), the K_m for cathepsin D was reported to range from 1.55 to 1.95 mg per ml (equivalent to 0.155 to 0.195 % hemoglobin respectively). This K_m value was close to a recently reported K_m of 0.19 % w/v hemoglobin based on a Lineweaver-Burk plot obtained over substrate concentrations of up to 2.5 % (Yamamoto et al., 1979). Doke et al. (1980) reported a K_m of 0.95 % hemoglobin

for cathepsin D isolated from the muscle of the fresh water fish Tilapia mossambicus

The activity of cathepsin D was recently found to be dependent on substrate concentration (Yamamoto et al., 1979). Maximal activity was observed at a hemoglobin concentration of 1.25 %, and concentrations above this level were inhibitory to the enzyme. This type of inhibition with hemoglobin as substrate was also shown in a cathepsin E like enzyme isolated by Yamamoto et al. (1978). Doke et al. (1980) observed that the activity of cathepsin D from the muscle of fresh water fish was linear at low concentrations and concentrations above 2 % were inhibitory to the enzyme. According to Yamamoto et al. (1979) the reasons for the inhibition are not known.

During cathepsin D activity assays, the concentrations of hemoglobin stock solutions have been variable. Stock solutions containing bovine hemoglobin at 2.5 % (Anson, 1938; Press et al., 1960; Yamamoto et al., 1978), 2 % (Okitani et al., 1981; Sliwinski et al., 1959; Robbins and Cohen, 1976), 1 % (Jasani et al., 1978; Doke et al., 1980; Kazakova and Orekhovich, 1976; Smith and Turk, 1974; Keilova and Tomasek, 1976), and 6 % (Iodice et al., 1966) have been used. This variability has also been reflected in the concentrations of hemoglobin in the final incubation mixtures with values invariably departing from the critical concentration of 1.25 % for maximum cathepsin D activity as reported by Yamamoto et al. (1979). Of more concern are the

activity assays which have been determined at substrate concentrations beyond the level for maximal cathepsin D activity as this may pose a technical problem in trying to compare activity assay data reported by cathepsin D researchers, in which case results may not be comparable due to lack of a standardized procedure. Following the mixing of the stock hemoglobin with an enzyme solution, water or some appropriate buffer, incubation mixtures have contained hemoglobin at 0.36 % (Okitani et al., 1981; Doke et al., 1980), 0.95 % (Kazakova and Orekhovich, 1976), 1 % (Sliwinski et al., 1959; Iodice et al., 1966; Kregar et al., 1967), 1.25 % (Yamamoto et al., 1979), 1.50 % (Press et al., 1960), and 1.67 % (Anson, 1938; Gianetto and de Duve, 1955; Smith and Turk, 1974; Keilova and Tomasek, 1976).

Cathepsin D Inhibition

Inhibition studies on cathepsin D and closely related acid proteinases have provided means of understanding the nature of the active site, the mechanism of catalysis, and of greater significance, application of suitable inhibitors as ligands for selective binding of the enzyme as a means of its purification by affinity chromatography. Cathepsin D is known to be inhibited by some inorganic and organic compounds.

Inhibition by Inorganic Compounds

Mercury and several bivalent metallic ions are potent inhibitors of cathepsin D (Kregar et al., 1967). Ferric and lead ions inhibited cathepsin DI and DII isolated from rat spleen (Yamamoto et al., 1979). Although the mode of inhibition by inorganic salts is not fully understood, the extent of inhibition depends on the type of salt and its concentration. The deleterious effect of inhibitory salts is also pronounced at the standard incubation temperatures. Anson (1938) reported that addition of ammonium sulfate to an incubation mixture at a rate of up to 0.001 M enhanced hemoglobin digestion. Concentrations greater than 0.01 % caused a sharp decrease in the rate of digestion. Woessner and Shamberger (1971) observed that salt solutions at ionic strengths exceeding 0.3 were inhibitory to cathepsin D isolated from bovine uterus. Recently Doke et al. (1980) reported that NaCl at 5 % and 10 % in a cathepsin D preparation caused a 60 % and over 80 % inhibition respectively after 2 h incubation at 37 C. Due to the inhibitory effects of ammonium sulfate and sodium chloride on cathepsin D proteolytic activity, fractions obtained following use of these salts (i.e for protein fractionation and prevention of nonspecific binding of proteins during purification by affinity chromatography respectively) have to be dialysed prior to activity assays.

Inhibition by Epoxy and Diazo Compounds

The action of epoxy and diazo compounds on acid proteinases has been extensively studied, especially the inhibitory action on pepsin (Tang, 1971; Chen and Tang, 1972; Tang et al., 1973; Huang et al., 1979; Lin and William, 1979). The two groups of compounds have also been reported to be inhibitory to cathepsin D (Turk et al., 1977; Huang et al., 1979). As mentioned earlier the epoxides and the diazo compounds interfere with the catalytic mechanism of pepsin and cathepsin D following esterification of Asp 32 and Asp 215, both residues being the active site for pepsin, cathepsin D and probably other proteinases.

Although the acid proteinases have similar groups participating in catalytic function, variability in substrate preference may arise due to differences in size and spatial orientation of the active site.

Inhibition by Pepstatin A

Pepstatin is a natural oligopeptide which was for the first time isolated from the culture media of Actinomyces longisporus flavus, Streptomyces argenteolus, and acid Streptomyces testaceus by Umezawa et al. (1970). Since its discovery pepstatin has been found to be a potent inhibitor of pepsin (Umezawa et al., 1970), human renin (McKown et al., 1974) and lysosomal cathepsin D (Dingle et al., 1972). It has proven to have medicinal application and is valuable

as a ligand for enzyme purification by affinity chromatography. Pepstatin has been used as a substitute for the antisera that prevents cartilage autolysis (Dingle et al., 1971). Its small molecular weight relative to the antisera used in prevention of cartilage autolysis is advantageous. Inhibitory tests on several common proteinases showed that pepstatin totally inhibited the acid proteases cathepsin D, cathepsin E, pepsin, renin, but had no action against neutral proteases trypsin, chymotrypsin, papain or cathepsin B1.

The exact mechanism by which pepstatin inhibits cathepsin D (Dingle et al., 1972; Knight and Barrett, 1976; Turk et al., 1977) and a few other proteinases including pepsin (Umezawa et al., 1970; Dingle et al., 1972) and human renin (McKown et al., 1974) is not fully understood. A mixed mode of inhibition of renin by pepstatin has been reported (Marks et al., 1973). Renin-pepstatin kinetic data independently analysed by Lineweaver-Burk, Hanes, Eadie-Hofstee, and Dixon-Webb methods indicated a reversible interaction between renin and pepstatin (McKown et al., 1974), and the inhibition was non competitive ($K_i = 1.3 \times 10^{-10}$ M). There are scanty kinetic data reported for pepstatin-cathepsin D interaction. According to Knight and Barrett (1976), one mole of pepstatin binds tightly to 43,000 grams of protein, a value which lies within the molecular weight range for cathepsin D. Binding of protein to pepstatin was in a 1:1 molar ratio. Knight and Barrett

(1976) therefore proposed the application of pepstatin as a titrant for accurate determination of the concentration of cathepsin D in solution. Whereas binding of pepstatin to cathepsin D is not readily reversible (Barrett and Dingle, 1972), renin-pepstatin interaction is readily reversible (McKown et al., 1974). On the other hand, analysis of cathepsin D-pepstatin kinetic data by a Dixon plot indicated the mode of inhibition as competitive (Lin and William, 1979).

The binding of pepstatin and its derivatives to cathepsin D has been shown to be pH dependent (Knight and Barrett, 1976). The apparent dissociation constant of a human cathepsin D-pepstatin complex was shown to increase from 5×10^{-6} M at pH 5.0 to 2×10^{-10} M at pH 6.4. According to Turk et al. (1977) the binding of pepstatin to cathepsin D as seen in circular dichroism (CD) spectra indicated that binding was within the acid range of 3.5 to 7.0 and there was absolutely no binding at and above pH 7.2. These results were in close agreement with an earlier report by Knight and Barrett (1976).

The site at which pepstatin binds to either porcine pepsin, human renin, cathepsin D or some fungal proteinases (Penicillium janthendum, Rhizopus chinensis, and Endothia parasitica) may be the same. The titration curve for cathepsin D showed an inflection point at pH 7.2 and the only amino acid titratable within this region is histidine. As pepstatin was incapable of binding cathepsin D at and

above pH 7.2 (Turk et al., 1977) it was inferred that binding takes place only when one of the histidine side chains is positively charged. Lin and William (1979) have also theorized that the unusual amino acid residue -4-amino-3-hydroxyl-6-methyl-heptyl or "statyl group" is responsible for the unusually strong inhibitory capability of pepstatin. "Statine" is an analog of the proposed transition state for catalysis by pepsin or other carboxyl proteinases. Similarity is thought to exist between the mode of binding of statine and that of competitive synthetic oligopeptide inhibitors having a Phe-Phe bond within their sequence.

Purification of Cathepsin D by Affinity Chromatography

The principle of purification of enzymes by affinity chromatography is that the enzyme to be purified is passed through a column containing crosslinked polymer or gel to which a competitive inhibitor or specific substrate has been covalently attached or immobilized (Guilford, 1973). All proteins with substantial affinity for a bound inhibitor or substrate will be retained in proportion to their affinity constant. Elution of the bound enzyme is achieved by changing such parameters as salt concentration, pH or addition of a competitive inhibitor to the solution (Cuatrecasas and Anfinsen, 1971a). Affinity chromatography has been successfully employed to purify staphylococcus nuclease, α -chymotrypsin and carboxypeptidase-A (Cuatrecasas

et al., 1968), renin (Murakani and Inagami, 1975) and cathepsin D (Smith and Turk, 1974; Barrett, 1972; Kazakova and Orekhovich, 1976; Gubensek et al., 1976; Benuck et al., 1977; Whitaker and Seyer, 1979a).

Generally a solid matrix such as sepharose is used as a support for the ligand to be immobilized. Sepharose is the trade name for a beaded form of a cross-linked dextran of highly porous structure manufactured by Pharmacia Fine Chemicals, Sweden. It is also known as agarose. The solid matrix covalently binds spacer arms bearing a terminal reactive functional group. The most commonly encountered functional group at the termini of spacer arms is either a carboxylic or an amino group. Sepharose with a terminal carboxylic group is known as "CH-Sepharose" and that with a terminal amino group is known as "AH-Sepharose or amino hexyl sepharose". Sepharose or any other solid support bearing a reactive functional group is made to react and form a covalent bond with a ligand to be immobilized. In order for such a reaction to take place, the carboxylic group which may either be on the solid support as in CH-Sepharose or on the ligand as in pepstatin must be activated before coupling can take place. The carboxylic group of pepstatin is activated either directly by water soluble carbodiimide (1-ethyl-3-dimethyl amino propyl) carbodiimide hydroxide (Murakani et al., 1973) or through the N-hydroxyl-succinimide ester in the presence of dicyclohexyl-carbodiimide and dimethylformamide as a coupling catalyst

(Murakani et al., 1973; Murakani and Inagami, 1975; Kazakova and Orekhovich, 1976).

Ligands that have been coupled to AH-Sepharose 4B include the cathepsin D substrate (Smith and Turk, 1974), a synthetic octapeptide inhibitory to cathepsin D (Gubensek, et al., 1976) and the natural oligopeptide pepstatin (Kazakova and Orechovich, 1976). The octapeptide used by Gubensek et al. (1976) was coupled by its terminal side chain which facilitated positioning of its active part at a distance from the solid matrix.

Pepstatin immobilized on AH-Sepharose was for the first time reported to have been used in the purification of chicken liver cathepsin D (Kazakova and Orekhovich, 1976). To date there have been very few reports on the use of immobilized pepstatin in purification of cathepsin D, including purification of bovine brain cathepsin D (Benuck et al., 1977; Whitaker and Seyer, 1979a) and muscle cathepsin D (Fan, 1981; Kregar et al., 1977). However, Okitani et al. (1981) were unable to purify muscle cathepsin D by affinity chromatography using pepstatin coupled to AH-Sepharose 4B.

Pepstatin is currently known to bind cathepsins D, E, and S. Although the three cathepsins are reactive towards the hemoglobin substrate, they can easily be distinguished from each other on the basis of the enormous differences in molecular weight, and this is easily achieved by electrophoretic resolution on SDS-polyacrylamide gels. The

molecular weight of cathepsins S, D, and E are 23,000 (Turk and Kregar, 1978), 40,000 (Smith and Turk, 1974) and 90,000 (Yamamoto et al., 1978; Jasani et al., 1978) respectively.

The affinity of pepstatin for cathepsin D is strongest at pH 3.5 and diminishes to zero as the pH is raised from 5.0 to 7.0 (Knight and Barrett, 1976; Turk et al., 1977). While binding of the sample is done at a pH in the region of 4.0, immobilized cathepsin D is eluted with a buffer of pH above 7. The alkaline buffer is introduced after exhaustive rinsing of the unbound proteins with an acidic buffer. The cathepsin D-ligand complex has been dissociated by elution with 0.15 M phosphate buffer, pH 8.0 (Gubensek et al., 1976) and 0.1 M tris-HCl buffer, pH 8.6 (Smith and Turk, 1974; Kregar et al., 1977; Fan, 1981). Sodium chloride is used in all column elution buffers to prevent nonspecific protein binding. In previous studies concentrations of sodium chloride in the elution buffers have been 0.5 M (Gubensek et al., 1976; Kazakova and Orekhovich, 1976) and 1 M (Smith and Turk, 1974; Kregar et al., 1977; Fan, 1981).

Studies Implicating Cathepsin D in Connective Tissue Breakdown

Incubation time

Woessner (1962) suggested that lysosomal particles contain enzymes capable of degrading connective tissue at an optimal pH within the acid range. These speculations were

confirmed by Frankland and Wynn (1962) who observed a sharp decline in viscosity coupled with release of free hydroxyproline from acid soluble collagen incubated with an extra-mitochondrial liver extract for less than 20 min, at pH 4.0 and 35 C. However the collagenolytic activity inherent in the liver extract was lost on repeating the experiment using an extract preincubated at 65 C for 40 min. The collagenolytic activity was not attributed to cathepsin C, since in a previous study by Tallan et al. (1952) cathepsin C retained its activity after similar incubation conditions. In a study by Laakkonen et al. (1970), the collagenolytic activity of a naturally occurring collagenase in meat was inactivated on cooking at temperatures above 60 C.

Woessner (1962) observed that rat uterus homogenate had a protease which was capable of digesting collagen and bovine hemoglobin at pH 3.5 and 37 C. The digestive action on collagen was not merely a solubilization effect involving breakage of a few bonds but rather a breakdown to the peptide stage. The degradation of collagen by proteases was proposed by Woessner (1962) as involving the following steps:-

i) Solubilization of collagen fiber at a rate proportional to fiber surface area.

ii) Thermal denaturation of solubilized collagen and hence its increased susceptibility to enzymatic attack.

iii) Attack of the collagen by lysosomal cathepsin with a concomitant release of peptides, and free amino

acids.

A lysosomal enzyme capable of liberating soluble hydroxyproline-containing products from insoluble collagen at an optimum pH of 3.45 was isolated from rat liver by Anderson (1969). The possibility of cathepsin A, B, C and E participating in such a hydrolysis was dismissed since the enzyme did not hydrolyse any of the synthetic substrates for trypsin under similar incubation conditions. Similar observations were made by Etherington (1972). However, the collagenolytic activity was inhibited by phenyl pyruvate. Phenyl pyruvate is inhibitory to cathepsin D (Barrett, 1967). Lysosomal extracts from several tissues indicated collagenolytic activity to be highest in spleen and kidney and relatively low in the ilium, muscle, skin and heart (Etherington, 1972). Cathepsin D activity had a similar distribution trend in the tissues. In a subsequent study by Burleigh et al. (1974), cathepsin D failed to degrade insoluble collagen at pH 3. Degradation of insoluble collagen was attributed to cathepsin B1 activity, at an optimum pH of 4.5 to 5.0.

Evidence from Studies on Involuting Uterus

Speculation that cathepsin D is involved in collagen hydrolysis have been based on studies relating its activity and the extent of collagen degradation in the course of time in involuting uteri postpartum. According to de Duve (1964),

during involution of mammalian uterus many tissue components including muscle protein, cellular material and connective tissue framework, particularly collagen, undergo resorption. According to Woessner (1965) the concentration of acid cathepsin and β -glucuronidase undergo a significant decrease during pregnancy and during postpartum involution they increase three to four fold with such increase being closely associated with a substantial decrease in collagen. The collagenolytic activity of the enzyme towards its substrate was increased on lowering the pH to around 3.5 which suggested a possible similarity between the specificity of the enzyme with that of bovine cathepsin D, as the uteri enzyme had a digestive action towards bovine hemoglobin at a pH optima of 3.5. Since uterine cathepsin D could be implicated in collagen breakdown only in an acid environment it was suggested that the breakdown takes place at the cell fiber interface or in the digestive vacuoles. The breakdown of collagen during uterine involution proceeds to the free amino acid level (Woessner and Brewer, 1963). Etherington (1973) however concluded that cathepsin D had no action on native insoluble collagen at pH 3.5 using extracts from rat uteri samples collected over a 12-day period postpartum.

Evidence from Studies on the Action of Cathepsin D on Cartilage

Cathepsin D has been implicated in degradation of proteoglycans which are the principal components of carti-

lage (Roughley and Barrett, 1977; Roughley, 1977). Weston et al. (1969) demonstrated that a specific antisera raised in rabbits and inhibitory to chicken and human cathepsin D inhibited chick cartilage degradation, the inhibition being up to 90 % (Dingle et al., 1971).

Further evidence that cathepsin D is involved in degradation of proteoglycans is based on the fact that pepstatin, which is a specific inhibitor of cathepsin D and related acid proteases, prevented human cartilage degradation (Dingle et al., 1972). The enzyme responsible for cartilage autolysis cleaved the β -chain of oxidized insulin (Woessner, 1973), a feature that distinguishes cathepsin D from the other cathepsins (Press et al., 1960).

The Role of Cathepsin D in Meat Tenderization

The principal components of muscle that influences tenderness are the myofibrillar proteins and the connective tissues (Winstanley, 1979). Numerous attempts have been made to correlate chemical and physical changes taking place in specific structural components of meat during postmortem aging. Modifications of connective tissue is desirable in reducing the toughness of meat since the nature and degree of connective crosslinks influence tenderness (Irvin and Cover, 1959; Goll et al., 1964a; Goll et al., 1964b). According to Wierbicki et al. (1955) and de Fremery and Streeter (1969) no changes have been observed in connective

tissue during aging of meat.

Shortly after death, while the pH is still near neutral (pH 7.0) calcium ions are released from the endoplasmic reticulum into the sarcoplasm where they activate a neutral protease called calcium activated factor (CAF) (Busch et al., 1972). It has been shown that meat treated with CAF loses all its Z-lines (Penny and Dransfield, 1979). As the pH of the carcass continues to fall towards a final pH of 5.5, the activity of the CAF decreases, and the increased acidification causes the lysosomes to rupture and with a concomitant release of lysosomal proteases into the sarcoplasm (Winstanley, 1979). The lysosomal proteases are active under acid conditions and two of them, cathepsins B and N, have been implicated in collagen breakdown (Etherington, 1976). It is probable that during meat aging, cathepsins B and N disrupt the crosslinking arrangements in collagen fibers. Cathepsin B has been implicated in the digestion of troponin T, one of the regulatory proteins of the thin filaments (Penny and Dransfield, 1979). It has been demonstrated that loss of troponin T parallels the increase in tenderization.

To date it is still debatable whether cathepsin D has a role in connective tissue degradation during meat aging. However "cathepsin D like" enzymes have been reported as having collagenolytic action (Anderson, 1969). Current evidence confirm cathepsin D to have degradative action against myofibrillar proteins. Early attempts to demonstrate cathe-

psin catalysed degradation of myofibrillar proteins were unsuccessful (Bodwell and Pearson, 1964; Martins and Whitaker, 1968). Most recent studies using the technique of sodium dodecyl sulfate polyacrylamide gel electrophoresis to monitor degradation have clearly shown that cathepsin B and D can degrade myofibrillar proteins (Schwartz and Bird, 1977). According to Schwartz and Bird (1977) the optimum pH for degradation of myofibrillar proteins by cathepsins B and D was pH 5.2 and 4.0 respectively. More recently Fan (1981) reported cathepsin D to degrade bovine myofibrillar proteins. Davey and Gilbert (1968a) and (1968b) showed that aging processes are associated with increased extractability of actin as well as disruption of Z-bands in the myofibrils.

The hypothesis that lysosomal cathepsin D is responsible for extracellular degradation of organic matrix of skeletal tissues is largely based on circumstantial evidence. True collagenases are active at pH above 6.0 (Anderson, 1969). The pH region within which collagenolytic action of lysosomal origin has been reported is 3.5 which is also the pH region that is optimal for cathepsin D activity. Enzymes that are responsible for collagen and proteoglycan breakdown are inhibited by pepstatin (Woessner, 1973; Dingle et al., 1972), and phenyl pyruvate (Barrett, 1967; Dingle et al., 1972). This is more convincing evidence that some of these enzymes are cathepsins. In addition to having either collagenolytic activity or degradative action on proteo-

glycans, the implicated enzyme(s) digest bovine hemoglobin at an optimal pH of 3.5 (Woessner, 1962; Woessner and Brewer, 1963), and are capable of cleaving the β -chain of oxidized insulin (Woessner, 1973). Similar characteristics are exhibited by cathepsin D. On the other hand Etherington (1972) reported that the collagenolytic cathepsin has negligible digestive action towards bovine hemoglobin.

The exact role which cathepsin D plays in degradation of connective tissue during meat aging is not fully understood. Studies of postmortem changes in collagen, the major component of connective tissue, has never revealed transformations comparable in extent to those which affect the myofibrillar structure during meat aging. Cathepsin D has been postulated to play a role in tenderization of meat with aging (Wu et al., 1981). Meat toughness has also been related to connective tissue content (Kim et al., 1967) and the degree of myofibrillar shortening (Winstanley, 1979; Chizzolini et al., 1977).

MATERIALS AND METHODS

Cathepsin D Extraction from Bovine

Sternomandibularis Muscle

Sternomandibularis muscle was obtained from a commercial grade carcass shortly after slaughter, and brought to the laboratory under ice. After being cut into smaller pieces weighing about 250 to 300 g, the pieces were stored at -10 C until required for enzyme extraction.

Portions of muscle to be extracted were thawed overnight at 4 C. Obvious connective tissue and adhering fat was removed with a sharp razor and the rest of the muscle chopped into small pieces with a pair of scissors. All the following steps were conducted at 0-4 C. The chopped pieces were homogenized in a high speed blender for 3 min after adding 4 times weight equivalents of ice cold 0.1 M KCl solution containing 1 mM sodium azide.

The homogenate was filtered through two layers of cheese cloth in order to trap large connective tissues and other debris. This was followed by centrifugation of the supernatant at 19,000 X G (12,800 rpm) for 30 min using an IEC International refrigerated centrifuge, model HR-1 (International Equipment Co., Boston, MASS.). The supernatant (hereafter referred to as "crude extract") was filtered through two layers of cheese cloth to remove fat

droplets and collected in a beaker submerged in an ice bath. While gently stirring with a magnetic stirrer, the supernatant was brought to 40 % saturation with ammonium sulfate by adding grade 1 purified crystalline ammonium sulfate (Sigma Chemical Company, St. Louis, MO.) at a rate of 22.6 g for every 100 ml of supernatant in accordance with ammonium sulfate saturation tables by Segel (1976). The 40 % ammonium sulfate-saturated supernatant was allowed to stand for 30 min in the ice/water bath after which it was centrifuged at 13,400 X G (10,800 rpm) for 30 min. The ammonium sulfate saturation of the supernatant was raised to 70 % by adding crystalline ammonium sulfate at a rate of 18.7 g for every 100 ml of the 40 % saturated supernatant (Segel, 1976). The 70 % ammonium sulfate extract was left to stand at 4 C for 30 min after which it was centrifuged at 13,400 X G for 30 min. Small volumes of 0.005 M sodium phosphate buffer, pH 8.0 were used to resuspend the sediment.

Dialysis Step

Five milliliter portions of the crude extract and the 40 % saturation supernatants together with the entire 40 to 70 % ammonium sulfate fraction were dialysed for 24 h against 0.005 M phosphate buffer of pH 8.0 using dialysis tubing of molecular cut off of 6,000 to 8,000 (Spectrum Medical Industries, Inc., Teminal Annex, Los Angeles, CA). The dialysis sediment was removed by centrifugation at 7,500 X G. The 40 to 70 % ammonium sulfate fraction was ready for

enzyme activity assay and further purification by affinity chromatography and ion exchange chromatography.

Extraction of Cathepsin D from Bovine Spleen

One hundred and thirty grams of bovine spleen were blended with 300 ml of ice cold water for 3 minutes. The homogenate was filtered through two layers of cheese cloth to remove large pieces of connective tissue. It was then centrifuged at 19,000 X G for 30 min. Ammonium sulfate fractionation leading to attainment of 40 to 70 % ammonium sulfate fraction was done following procedures used for extraction of muscle cathepsin D. The solution of the 40 to 70 % ammonium sulfate sediment in 0.005 M sodium phosphate buffer, pH 8.00 was dialysed overnight against the same buffer. The dialysis sediment was removed by centrifugation after which the aliquot was further purified by affinity chromatography.

Purification of Bovine Cathepsin D by Affinity Chromatography

Preparation of Affinity Chromatography Column

An affinity chromatography column was prepared following the principles for preparation of an AH-Sepharose 4 B-pepstatin column (Kazakova and Orekhovich, 1976). Controlled glass support beads with long alkylamine spacer arms (Pierce

Chemicals, Rockford, IL., U.S.A) were used in this study.

Activation of Pepstatin

Thirty milligrams of pepstatin A (Sigma Chemical Company, St. Louis, MO.) were dissolved in 3 ml of dimethylformamide followed by addition of 3.4 mg of N-hydroxylsuccinimide. In a second beaker, 20 mg of dicyclohexylcarbodiimide were dissolved in 1 ml of dimethyl formamide. Both solutions were cooled to 4 C after which they were mixed and allowed to stand at 4 C for 1 h.

Preparation of the Glass Beads

Two grams of glass beads were suspended in 10 ml of dioxane and shaken. The dioxane was decanted from the beads and then washed twice with dioxane, and finally with 500 ml of deionized water.

Coupling of Activated Pepstatin to the Glass Beads

The activated pepstatin solution (about 4 ml) and 8 ml of a 1:1 mixture of 0.1 M phosphate buffer (pH 6.1), and dimethylformamide were added to the glass bead slurry. The reaction mixture was stirred gently at room temperature for 18 h, in a small beaker securely covered with parafilm to exclude atmospheric moisture. After the reaction, the aliquot was decanted off trapping any suspended beads on Whatman filter paper number 3. The residue was further

washed four times with 6 ml portions of dimethylformamide.

Blocking Unreacted Amino Groups on the Glass Beads

Unreacted amino groups were blocked with activated acetic acid as suggested by Murakani et al. (1973). The principle of activation was similar to that used for activation of pepstatin. Ten millimoles of acetic acid (0.6 g or 0.57 ml of glacial acetic acid) and 3.4 mg of hydroxysuccinimide were dissolved in 3 ml of dimethylformamide. In a separate beaker, 20 mg of hexylcarbodiimide (Sigma Chemical Company, St. Louis, MO.) were dissolved in 1 ml of dimethylformamide. The two solutions were separately cooled to 4 C before mixing. The activation reaction was allowed to proceed for 30 min in an ice bath. The solution of activated acetic acid was then added to the glass beads-pepstatin along with 10 ml of a 1:1 mixture of dioxane and 0.1 M phosphate buffer (pH 7.4). The mixture was stirred for 2 h at room temperature. The supernatant was decanted off. Three 20 ml portions of a 1:2 mixture of dioxane and dimethylformamide were used for the initial washing of the beads.

The slurry was washed twice with 50 ml portions of each of two buffers (0.1 M sodium acetate and carbonate buffers of pH 4.0 and 8.6 in 0.5 M sodium chloride, respectively) to displace ionic impurities. The slurry was then loaded onto a chromatographic column (Bio-Rad Laboratories, Richmond, CA.) (1.0 x 10 cm) to a 5.0 cm height and finally fitted with a

flow adapter. The column was equilibrated overnight by circulating 0.1 M sodium citrate buffer, pH 3.9.

Affinity Chromatography
of Cathepsin D from bovine
muscle and spleen

Nine milliliters of previously prepared 40-70 % ammonium sulfate fraction was acidified to pH 4.0 by mixing with an equal volume of 0.08 M sodium citrate buffer at pH 3.2 (Benuck et al., 1977). The resulting precipitate was removed by centrifuging at 7,500 G for 20 min. The clear supernatant was circulated through the affinity chromatography column for 45 min.

The unbound protein eluted with 0.1 M sodium acetate buffer, pH 4.0. The pepstatin-bound protein was eluted with 0.1 M sodium carbonate buffer, pH 8.6. Both elution buffers contained 0.5 M sodium chloride. The eluate absorbance at 280 nm was monitored and recorded with a Model UA 5 absorbance monitor (Instrumentation Specialities Company, Lincoln, NE.). One milliliter fractions were collected by a Model 1200 Fraction collector (ISCO, Lincoln, NE.).

The pH 8.6 eluate fractions which contained the cathepsin D peak were pooled and dialysed for 24 h against 0.005 M sodium phosphate buffer, pH 8.00 to remove sodium chloride. The dialysed fraction (about 10-12 ml) was concentrated to about 3.0 to 4.0 ml under an atmosphere of nitrogen using Y-5 Diaflo ultrafiltration membranes (Amicon Corporation, Lexington, MASS.).

Before reusing the column, it was washed with 4 M urea, then re-equilibrated with 0.1 M sodium citrate buffer, pH 3.9.

Purification of Muscle Cathepsin D by
Ion Exchange and Gel Filtration
Chromatography

A trial was made to further purify the 40 to 70 % ammonium sulfate fraction by ion exchange chromatography and gel filtration. The fraction was dialysed against 0.01 M sodium phosphate buffer, pH 7.0. The dialysis precipitate was removed by centrifugation. Five milliliter portions of the dialysed 40 to 70 % saturated ammonium sulfate fraction were loaded on a freshly prepared diethyl aminoethyl-sephadex A-50 (DEAE-Sephadex A-50) column equilibrated with 0.01 M sodium phosphate buffer, pH 7.0. The starting bed volume was 46 ml. The column was eluted, first by 0.1 M sodium phosphate buffer to wash out unadsorbed proteins, followed by elution with 0.1 M sodium phosphate buffer containing 0.14 M sodium chloride to elute the fraction containing cathepsin D activity (Okitani et al., 1981). Both elution buffers were at pH 7.0. The fractions eluted by 0.1 M phosphate buffer containing 0.14 M sodium chloride were pooled and concentrated to 5-8 ml by membrane ultrafiltration. The concentrated fraction was further purified by gel filtration on Sephadex G-100 equilibrated with 0.01 M sodium phosphate buffer, pH 6.0. The column was eluted with

the same buffer, collecting 2 ml fractions.

Cathepsin D Activity Assays

Muscle Cathepsin D Activity Assay

The optimum pH for muscle cathepsin D activity was established using urea-denatured 2 % hemoglobin as substrate. Urea denatured hemoglobin was prepared by incubating a 2 % hemoglobin solution containing 3 M urea at 37 C for 1 h.

Assays for bovine muscle cathepsin D activity were run at pH 3.2, 3.5, 3.8, and 4.0 using a 40 to 70 % saturated ammonium sulfate fraction. A 2 ml incubation volume contained 1.0 ml of 0.2 M sodium formate buffer (of either pH 3.2, 3.5, 3.8 or 4.0), 0.5 ml of the 2 % urea denatured bovine hemoglobin solution, and 0.5 ml of the enzyme solution. Blanks consisted of enzyme solution in the buffers and 1 ml of 2 % hemoglobin, incubated separately according to the method of Robbins and Cohen (1976). After 1 h incubation at 37 C, 3 ml of 10 % ice cold trichloroacetic acid (TCA) were added to precipitate proteins. Three milliliters of TCA were also added to the blank preparation followed by 1 ml of 2 % hemoglobin solution incubated for the same period. Tyrosine-bearing peptides in the TCA filtrate were determined by the method of Lowry et al. (1951) as modified by Smith and Turk (1974). All subsequent muscle cathepsin D activity assays were performed at pH 3.8.

Spleen Cathepsin D Activity Assay

Spleen cathepsin D activity was assayed using a 2 % bovine hemoglobin prepared by dissolving crystalline hemoglobin in a 0.15 M sodium citrate buffer, pH 2.8. The hemoglobin solution was filtered through four layers of cheese cloth followed by centrifugation at 1800 X G for 20 min. The pH of the resulting solution was 3.2.

An incubation time-activity profile for bovine spleen cathepsin D was determined by incubating a 2 % hemoglobin solution with the enzyme as previously described, but in a larger volume. Duplicate samples were drawn at 0, 10, 20, 30, 40, 50, 60, and 70 min and transferred to 3.0 ml of ice cold 10 % TCA.

Also, a pH-activity assay for bovine spleen cathepsin D was tested over the pH range 3.0 to 4.5 using an extract purified by affinity chromatography. The pooled fractions representing the cathepsin D peak in the pH 8.6 eluate were dialysed for 36 h before activity assay as described earlier. Incubation mixtures consisted of 0.4 ml of the dialysed, unconcentrated eluate, 0.5 ml of 2 % hemoglobin substrate, 1.0 ml of 0.1 M sodium formate buffer (of either pH 3.0, 3.5, 4.0 or 4.5) and 0.1 ml of water, to bring the assay volume to 2.0 ml. After 30 min incubation at 37 C, 3 ml of ice cold 10 % TCA was added. The sample was filtered, and tyrosine equivalents of the TCA soluble peptides determined by the procedure of Lowry et al. (1951). As a result

of these preliminary studies, all subsequent spleen cathepsin D activity assays were conducted at pH 3.2 and 37 C for 30 min.

The hemoglobin solution was preincubated at 37 C before being added to the enzyme preparation. The enzyme preparation consisted of 0.05 ml of either the 40 to 70 % saturated ammonium sulfate fraction or the concentrated affinity chromatography peak (for unconcentrated preparations, 0.4 ml of affinity fraction was used), each made to 1.0 ml with distilled water. The crude extract (0.1 ml) was diluted to 1.0 ml before addition of 1.0 ml of the substrate solution. Blanks were prepared according to the procedure by Robbins and Cohen (1976).

After protein precipitation by TCA, the samples were filtered through 2 layers of Whatman number 42 filter paper. The TCA filtrate (0.5 ml) was mixed with 1.0 ml of 0.5 M sodium hydroxide and 0.3 ml of diluted phenol reagent (Folin & Ciocalteu reagent diluted with water in a 1:3 ratio). The absorbance of the blue color formed was read at 750 nm against an appropriate blank within 5 to 10 min.

Preparation of L-tyrosine Standard Curve

A 10 micromole per ml L-tyrosine stock solution was prepared by dissolving 0.1821 g of L-tyrosine (Calbiochem-Behring Corp. La Jolla, CA.) in 100 ml of 6 % TCA. Standard solutions containing 0.0072, 0.0180, 0.0360 and 0.0570

μM tyrosine per ml were prepared by diluting the stock solution. After addition of 1.0 ml of 0.5 N sodium hydroxide solution and 0.3 ml of diluted phenol reagent (Smith and Turk, 1974) the standards contained 0.002, 0.005, 0.010, and 0.015 μM tyrosine per ml respectively (APPENDIX A, Table 6). An L-tyrosine standard curve was prepared (APPENDIX A, Figure 16).

Cathepsin D activity values were calculated using the following sequence of equations:-

$$\begin{aligned}
 &1. \quad \begin{array}{l} \mu\text{M L-tyrosine} \\ \text{released per} \\ \text{per unit time} \end{array} = \left(\frac{A_{750} - Y \text{ intercept}}{\text{slope}} \right) \\
 & \quad \quad \quad \mu\text{M L-tyrosine per ml in the} \\
 & \quad \quad \quad \text{reaction mixture} \\
 & \quad \quad \quad \times 1.8 \text{ ml reaction mixture} \\
 & \quad \quad \quad \times 5.0 \text{ ml of volume of TCA} \\
 & \quad \quad \quad \text{filtrate} \\
 & \quad \quad \quad / 0.5 \text{ ml TCA filtrate.} \\
 \\
 &2. \quad \text{Activity} \quad = \quad \mu\text{M L-tyrosine} \quad \text{Volume of enzyme} \\
 & \quad \quad \quad (\text{units/ml}) \quad = \quad \text{released per} \quad / \quad \text{added to the assay} \\
 & \quad \quad \quad \quad \quad \quad \text{unit time} \quad \quad \quad \text{mixture} \\
 \\
 & \quad \quad \quad \mu\text{M L-tyrosine released} \\
 & \quad \quad \quad \text{per unit time} \\
 & \quad \quad \quad = \frac{\text{-----}}{\text{ml of Enzyme solution}}
 \end{aligned}$$

A_{750} = Absorbance at 750 nm.

Y intercept = 0.006 Absorbance units (Figure 16).

Slope = 13.73 Absorbance / μM L-tyrosine/ml (Figure 16).

Protein Determination

Protein was determined by the method of Lowry et al. (1951). Lowry's solutions A (20 g anhydrous sodium carbonate, 4.0 g sodium hydroxide, and 0.2 g of sodium potassium tartarate tetrahydrate per liter) and B (6 g cupric sulfate pentahydrate per liter) were mixed in a ratio of 50:1 to form Lowry's solution C. The crude extract and the 40 to 70 % saturated ammonium sulfate saturation fraction were diluted 50 and 200 fold respectively before being assayed for protein. Samples of the crude extract (0.2 ml) and the 40-70 % saturated ammonium sulfate saturation fraction (0.05 ml) were diluted to 10 ml. The concentrated pH 8.6 eluate was assayed for protein without further dilution.

To 0.2 ml of the diluted sample, 1.0 ml of Lowry's solution C was added, and left to stand for 10 min. Then 0.1 ml Folin & Ciocalteu reagent (diluted with 5 % sulfuric acid in a 1:1 ratio) was added and immediately mixed. The assay mixtures (in triplicate) were left to stand for 30 min for color development. Blanks consisted of Lowry's solution C, diluted Folin's & Ciocalteus phenol reagent and 0.2 ml of deionized water. Absorbance was determined at 660 nm against respective blanks. Protein concentrations were evaluated from a bovine albumin standard curve.

Bovine albumin stock solution (650 $\mu\text{g/ml}$) was prepared by diluting 542 μL of 30 % bovine albumin (Calbiochem-Behring Corp., La Jolla, CA.) to 250 ml. Standard protein

solutions were prepared by diluting 1, 2, 3, and 4 ml of the stock solution each to 10 ml. Following addition of all reagents, the assay mixtures contained 10, 20, 30 and 40 μ g protein per ml. A bovine albumin standard curve was prepared (APPENDIX B, Figure 17).

SDS-Gel Electrophoresis of the pH 8.6 Eluate

The molecular weight of the proteins in the concentrated pH 8.6 eluate were evaluated by performing SDS-polyacrylamide gel electrophoresis using 10 % gels following the procedure of Porzio and Pearson (1977). A mixture sufficient for preparation of 16 gels consisted of 13.6 ml of 25 % acrylamide, 0.25 % bisacrylamide; 6.6 ml of 2.0 M tris-glycine buffer (0.5 M tris, 1.5 M glycine, pH 8.6); 2.0 ml of glycerol; 1.3 ml of 2.5 % sodium dodecyl sulfate (SDS); 13 μ L of tetramethyl ethylene diamide (TEMED); 9 ml of distilled water; and 1.3 ml of 1 % ammonium persulfate. The mixture was stirred gently, to avoid incorporation of oxygen and immediately dispensed by syringe into gel tubes (80 x 6 mm i.d.) to a height of 7.5 cm. The bottoms of the tubes had been temporarily sealed with a paraffin film (held in place by rubber caps) in order to prevent loss of the reaction mixture prior to polymerization. The meniscus at the top of the gels was eliminated by carefully introducing a layer of distilled water. Polymerization was completed within half an hour. The paraffin film and the supporting rubber stoppers

were removed from the bottom of the tubes and the gel tubes were fitted to the tube holder of an Electrophoresis Cell (Bio-Rad Model 150 A, Richmond, CA.). The bottom chamber of the electrophoresis cell was half filled with about 750 ml of chamber buffer (0.1 M tris-glycine buffer of pH 8.6, containing 0.1 % SDS). About 200 μ L of the concentrated pH 8.6 eluate and a solution of protein standards were mixed with equal volumes of a tracking dye preparation (25 ml of which was prepared by dissolving 0.0050 g of pyronin Y, 0.0039 g of dithiothreitol and 0.215 g of ethylene diamine tetraacetic acid (EDTA) in a mixture of 10 ml of 2.5 % SDS, 0.625 ml of 2.0 M tris-glycine buffer of pH 8.6, 5.0 ml of glycerol, and 9.25 ml of distilled water) and boiled in a water bath for 5 min. Protein denaturation was enhanced by the presence of dithiothreitol in the tracking dye preparation. The mixture of protein standards (Bio-Rad Laboratories, Richmond CA.) consisted of phosphorylase b (MW= 92,500), bovine serum albumin (MW= 66,200), ovalbumin (MW= 45,000), carbonic anhydrase (MW= 31,000), soybean trypsin inhibitor (MW= 21,500) and α -lactalbumin (MW= 14,400). Each sample and the mixture of protein standards (50, 100, and 150 μ L sample volumes) were carefully layered on the top (cathode) of the gels. Direct current was supplied by a HEATHKIT Regulated High Voltage power supply Model IP-2717 (Heath Company, Benton Harbor, MI.) at a rate of 1.0 mA during the first hour after which the rate was increased to 2.0 mA per gel. After the tracking dye had reached a di-

stance of about .5 cm from the tube bottom (i.e. after a period of 3.0 hours of total run), the power was disconnected and the gels were removed from the chamber.

A syringe filled with chilled water was inserted along the gel periphery while injecting a controlled stream of water to force the gels out of the tubes. The positions reached by the tracking dye were marked by insertion of a syringe needle. Finally the gels were stained overnight in a staining solution. A staining solution sufficient for staining 12 gels consisted of 0.05 g coomassie brilliant blue R in a mixture of 75 ml of methanol, 10.5 ml of glacial acetic acid and 64.5 ml of distilled water.

Destaining was done in an electrophoresis diffusion destainer (Bio-Rad Model 172 A) filled with 3,500 ml of 7.5 % (v/v) glacial acetic acid 5 % methanol (v/v) in distilled water. After destaining for a period of 48 to 72 h, the lengths of the gels were measured and recorded. The relative electrophoretic mobilities (Rf) of the protein standards and those of protein bands resolved from the pH 8.6 eluate were calculated using the formula proposed by Weber and Osborn (1969):

$$Rf = \frac{(\text{Prestained gel length})}{(\text{Destained gel length})} \times \frac{(\text{Band distance})}{(\text{Dye distance})}$$

A semilogarithmic plot of molecular weight of protein standards (APPENDIX C, Table 7) and their corresponding relative electrophoretic mobilities (Rf) was constructed.

The molecular weights of the unknown proteins were determined from the molecular weight standard curve (APPENDIX C, Figure 18). For long term storage, gels were kept in 10 % glacial acetic acid.

Studies on Connective Tissue

Connective Tissue Isolation

Intramuscular connective tissue (IMCT) was isolated from beef shank muscle by procedures based on the method of Wu et al. (1982). Four hundred grams of muscle were blended two times in 2,000 ml of icecold deionized water for 1.5 min, followed by filtration of the homogenate through plastic cheese cloth (The Bandage Corporation, Sheboygan WIS.) to trap connective tissue. The connective tissue was further blended three times in 1,000 ml portions of 0.1 M sodium phosphate buffer (pH 7.0) containing 1.1 M KCl, and connective tissue was trapped by filtering through cheese cloth. Traces of myofibrils were removed from the connective tissue pellet by washing in a large volume of 1 M KCl. Finally potassium ions were removed by washing in 0.9 % NaCl solution. The purified connective tissue was freeze-dried and ground to a fine powder by a CORONA manual grinding machine (Landers & Scia. S. A., Denver, COL.).

Isolation of Acid Soluble Collagen

Acid soluble collagen was extracted by the method of Gibson et al. (1978). One gram of the freeze dried bovine shank intramuscular connective tissue was suspended in 50 ml of 0.2 M glacial acetic acid and vigorously stirred for 36 h at 4 C. The suspension was filtered through Whatman number 3 paper and the filtrate dialysed against 0.005 M sodium phosphate buffer, pH 8.0. The filtrate was then ready for analysis by SDS-polyacrylamide gel electrophoresis using 7.5 % gels.

Studies on the Action of Cathepsin D and Pineapple stem Bromelain on Intramuscular Connective Tissue

The digestive action of bovine cathepsin D (from spleen and muscle) on intramuscular connective tissue, IMCT, (isolated from shank muscle) was tested at pH 3.0, 3.5, 4.0 and 4.5. The bovine muscle and bovine spleen cathepsin D were preparations purified by ion-exchange chromatography and affinity chromatography respectively. The specific activity of the bovine muscle and bovine spleen cathepsin D were 0.453 and 39.8 units per mg protein respectively. The enzyme solution (0.5 ml for the muscle extract, or 0.4 ml of the spleen extract) were used.

For each pH level, 100 mg of intramuscular connective tissue sample was suspended in three test tubes each con-

taining 5 ml of 0.1 M sodium formate buffer. One milliliter of pepstatin A solution in 0.005 M NaOH (prepared by dissolving 17.5 mg of pepstatin A in 20 ml of 0.005 M NaOH) was added to one of the tubes. The volume was made to 10 ml by adding 1.5 and 2.5 ml of distilled water to the test tube with and without pepstatin respectively. The third test tube served as a second control, containing 100 mg of IMCT, 5 ml of 0.1 M sodium formate buffer, and 5 ml of distilled water.

The test tubes were transferred to a water bath at 37 C. The first set of 2 ml samples (representing zero incubation time) were drawn within the first 30 min of incubation. Two more sets of samples were drawn at 8 and 24 h of incubation. The 2 ml samples were filtered through Metricel membrane millipore filters of 0.45 μ m pore size and 25 mm diameter (Gelman Sciences, Inc, Ann Arbor MI.) The filtrate was analysed for free hydroxyproline, and total collagen.

The above test was repeated using commercial preparations of bovine spleen cathepsin D (Sigma Chemical Company, St. Louis, MO.) and pineapple stem bromelain (Calbiochem-Behring Corp., La Jolla, CA.). The incubation mixtures consisted of either 0.45 bovine spleen cathepsin D units (Sigma Chemicals) or 0.1 % pineapple stem bromelain in an incubation volume of 10 ml.

Determination of Hydroxyproline

Hydroxyproline was determined by the method of Dahl and Persson (1963). Buffered chloramine T was prepared by

mixing 100 ml of chloramine T (2.82 g sodium N-chloro-p-toluene sulfonamide solution in 40 ml of water made to 100 ml by adding methyl cellosolve) with 100 ml of acetate-citrate buffer pH 6.0 (10 g of citric acid monohydrate, 2.4 ml of glacial acetic acid, 24 g of sodium acetate and 6.4 g of NaOH, all dissolved in water).

Standard hydroxyproline solutions were prepared by diluting 0.2, 0.4, 0.6, and 0.8 ml of a hydroxyproline stock solution (30 mg of L-hydroxyproline dissolved in 1 liter of 0.001 M HCl) to 2.0 ml followed by addition of 1 ml of chloramine T, 2 ml of 4 M perchloric acid (prepared by mixing 133 ml of 70 % perchloric acid with 233 ml of distilled water) and 1 ml of 20 % w/v p-dimethylaminobenzaldehyde solution in methyl cellosolve. The reaction mixtures were allowed to stand at room temperature for 20 and 10 min following addition of chloramine T and perchloric acid respectively. After addition of p-dimethylaminobenzaldehyde, color was developed by incubating the reaction mixture at 60 C for 15 min. Absorbance was determined at 550 nm against a blank consisting of 2 ml of 0.001 M HCl, 1 ml of chloramine T solution, 2 ml of 4 M perchloric acid and 1 ml of 20 % p-dimethylaminobenzaldehyde. The final reaction mixture contained 1, 2, 3, and 4 mg hydroxyproline per liter respectively. A hydroxyproline standard curve is shown in APPENDIX D, Figure 21.

Determination of Free Hydroxyproline in IMCT Incubated with Enzyme

To 0.4 ml of each of the sample and control filtrates, 0.2 ml of chloramine T solution was added. After incubation at room temperature for 20 min, 0.4 ml of 4 M perchloric acid was added. After a further 10 minutes, red color was developed by adding 0.2 ml of p-dimethylaminobenzaldehyde followed by incubation at 60 C for 15 min.

Analysis for Total Collagen in IMCT Samples Incubated with Enzyme

The filtrates were further analysed for total collagen by hydrolysing 0.2 of the filtrate with 0.8 ml of 7.5 M HCl in sealed tubes placed in a pressure cooker at 110 C for 2 h to hydrolyse soluble collagen to amino acids. Two 20 mg samples of purified connective tissue were also hydrolysed by adding 1.0 ml of 6.0 M HCl. One ml of distilled water was added to each of the tubes. Then free hydroxyproline was determined as previously described. The collagen content was computed by multiplying the hydroxyproline content by a factor of 7.46 (Woessner and Brewer, 1963).

SDS-Polyacrylamide Gel Electrophoresis of Connective Tissue Digests

Samples which were positive for hydroxyproline in connective tissue digests were further analysed by SDS-

polyacrylamide gel electrophoresis using 7.5 gels prepared by the method of Furtmayr and Timpl (1971).

Reagent solutions enough to fill 24 gel tubes consisted of 25 ml of solution A (3.75 g of acrylamide, 0.100 g of bis-acrylamide and 25 microliters of TEMED dissolved in 0.1 M phosphate buffer of pH 8.0 containing 0.1 % SDS) and solution B (0.0375 g of ammonium persulfate dissolved in the same buffer used for preparation of solution A). Gel polymerization was initiated by mixing equal volumes of solutions A and B.

Sample Preparation

Two hundred microliters of concentrated hydrolysates were mixed with 100 μ L of a special tracking dye preparation (20 ml of which contained 0.6 g of SDS, 0.6 ml of mercaptoethanol, 0.004 g of pyronin Y, 4 ml of glycerol, and 14.5 ml of distilled water). The sample dye mixture contained 1 % SDS and 1 % mercaptoethanol as recommended by Slykes and Bailey (1971). Sample dye mixture was incubated at 37 C for 2 h. Acid soluble collagen samples were prepared similarly.

Protein standards, and a 1 % solution of pineapple stem bromelain (dissolved in water and filtered through Whatman number 3 filter paper) were mixed with the tracking dye preparation used previously for gel electrophoresis of globular proteins. The mixtures were boiled for 5 min.

The samples were applied to the gels and a current of 5 mA per gel was applied for 7 h. The gels were stained in

a 0.05 % solution of coomassie brilliant blue R in a mixture of 40 % methanol and 1 M acetic acid. The stained gels were destained for 24 hours in 20 % methanol, 1 M acetic acid, then stored in 10 % glacial acetic acid.

RESULTS AND DISCUSSION

General Observations on Procedures for
Purification of Bovine
Cathepsin D

The procedures used in this study for purification of bovine muscle and spleen cathepsin D up to the stage of obtaining a 40-70 % ammonium sulfate fraction have previously been reported by Smith and Turk (1974), Kregar et al. (1977) and Fan (1981). In all cases, the 40-70 % fraction was reported to have been further purified by affinity chromatography on either hemoglobin or pepstatin coupled to AH-Sepharose 4B.

In this study, the AH-Sepharose 4B-pepstatin column performed poorly in purification of bovine muscle cathepsin D. Therefore, glass beads bearing terminal amino groups (Controlled pore glass beads bearing long alkylamine, Pierce Chemicals, Rockford, IL.) were for the first time tested as a support for coupling pepstatin, under reaction conditions that were similar to coupling of pepstatin to AH-Sepharose as previously reported by Murakani et al. (1973) and Kazakova and Orekhovich (1976).

Affinity chromatography elution profiles of acidified 40 to 70 % ammonium sulfate fractions using a glass bead-pepstatin column are shown in Figures 1 and 2. The elution patterns in Figures 1 and 2 represent affinity purification

Figure 1. Protein as measured by absorbance at 280 nm on bovine muscle fractions eluted from glass bead-pepstatin affinity column. Seven milliliters of 40-70 % ammonium sulfate fraction were loaded and 1 ml fraction collected at a column flow rate of 15 ml per hour. The column was first eluted with 0.1 M sodium acetate buffer, pH 4.0 to wash the unbound proteins represented by fractions 1-25. The arrow indicates change of elution buffer to 0.1 M sodium carbonate buffer, pH 8.6. Both elution buffers contained 0.5 M sodium chloride. Fractions 31-42 were pooled and concentrated to 2.0 ml.

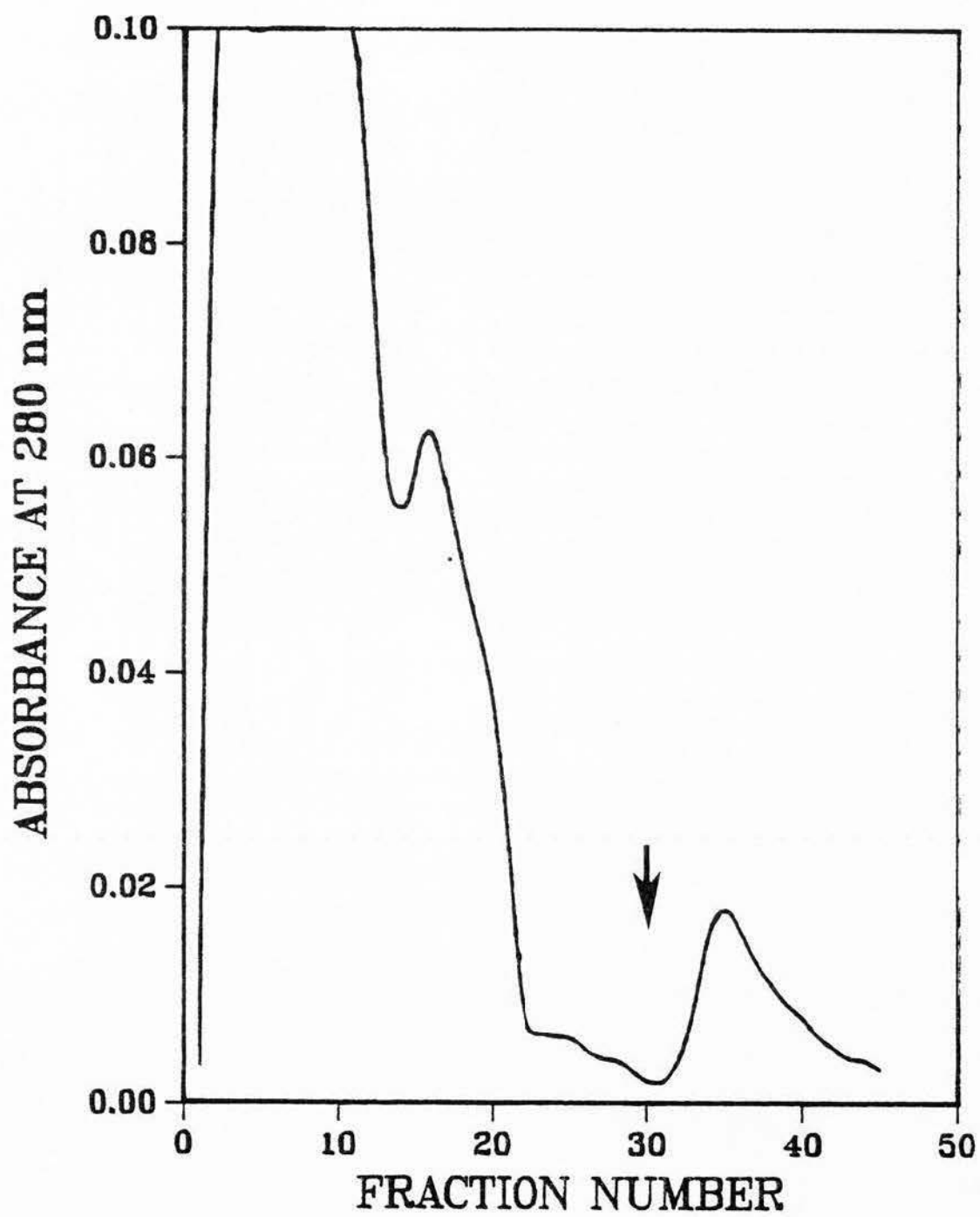
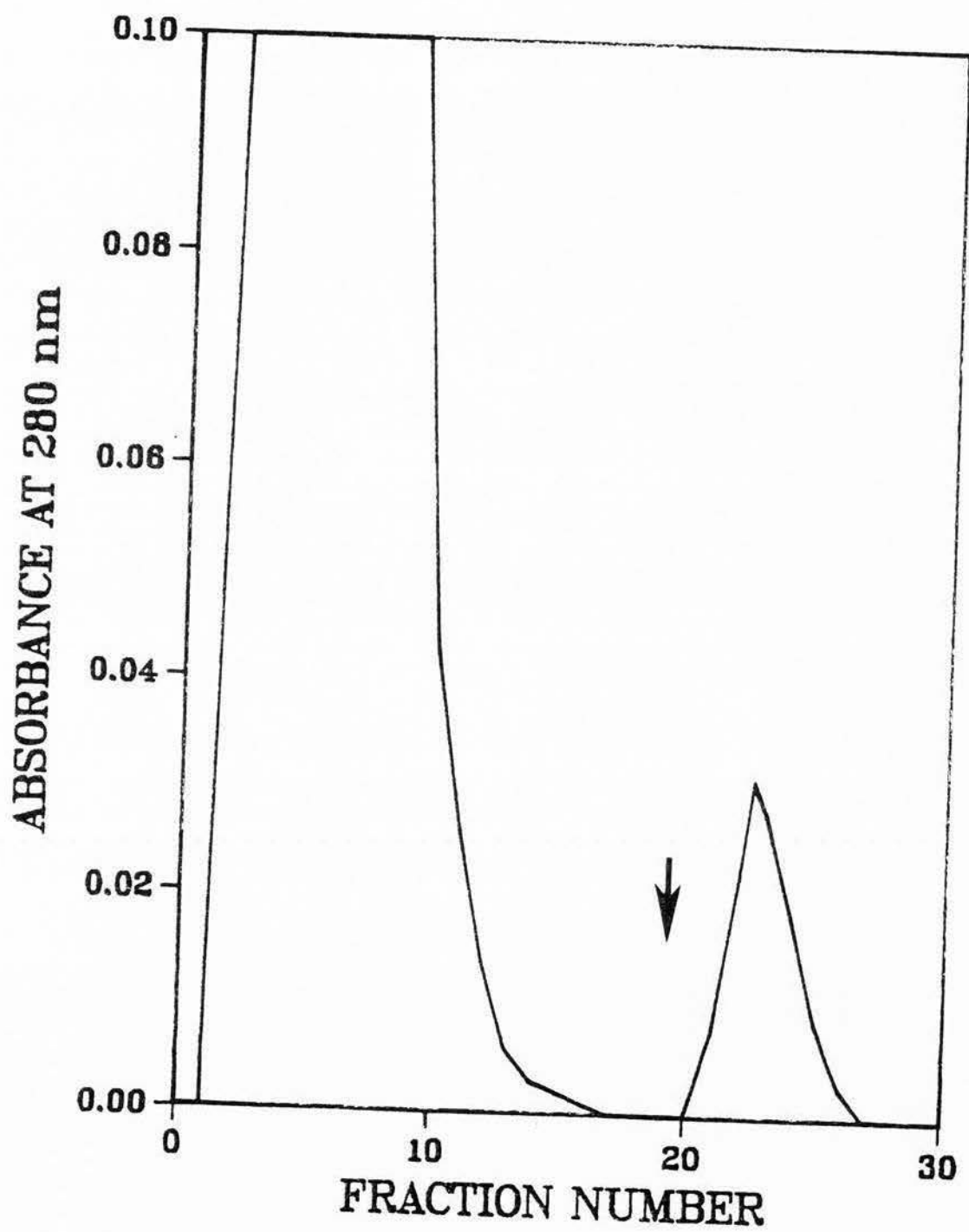


Figure 2. Protein as measured by absorbance at 280 nm on bovine spleen fractions eluted from a glass bead-pepstatin column. Ten milliliters of the 40-70 % ammonium sulfate fraction of bovine spleen were loaded. The unbound proteins were washed from the column with 0.1 M sodium acetate buffer, pH 4.0 and then the immobilized proteins were eluted by changing the elution buffer to 0.1 M sodium carbonate buffer, pH 8.6 as indicated by the arrow. Both elution buffers contained 0.5 M sodium chloride. One milliliter fractions were collected. The column flow rate was 10 ml per h. Fractions 21-28 were pooled and represented the immobilized cathepsin D.



of bovine muscle and spleen cathepsin D respectively, and are identical to those previously reported for agarose-hemoglobin (Smith and Turk, 1974) and agarose-pepstatin (Murakani et al., 1973) columns. The profiles show two major peaks. The second peak in both cases represents protein that was immobilized by pepstatin A.

Previous studies have indicated that binding of cathepsin D by pepstatin A and its derivatives is strongly pH dependent (Knight and Barrett, 1976). The dissociation constant for the complex between pepstatin and human cathepsin D was found to be considerably increased as the pH was raised from 5.0 towards neutrality. Gubensek et al. (1976) reported negligible binding of cathepsin D by pepstatin coupled to AH-Sepharose 4B at pH above 4.5. According to Knight and Barrett (1976), pepstatin A has maximum affinity for cathepsin D at pH 3.5. Apparently, the affinity chromatography purification studies previously reported by Kregar et al. (1977) and Fan (1981) did not take into serious consideration the pH effects on the extent of immobilization of cathepsin D. In these studies muscle cathepsin D was immobilized on an affinity column pre-equilibrated with a buffer of pH 5.0, and using the same buffer for the first stage elution of the column. Elution of the column at pH 5.0 is likely to result in a substantial loss of cathepsin D in the pH 5.0 eluate with minimum enzyme recovery on changing the elution buffer to one having a pH within the alkaline range. This argument is based on the previously reported

minimum interaction between the ligand pepstatin and cathepsin D at pH 5.0 (Knight and Barrett, 1976; Turk et al., 1977). Okitani et al. (1981) also reported a failure in attempts to purify muscle cathepsin D on AH-Sepharose 4B following the procedure of Smith and Turk (1974) and Kregar et al. (1977). Although pepstatin is reported to have maximal affinity for cathepsin D at pH 3.5 (Knight and Barrett, 1976), the enzyme is also very unstable at such low pH, especially in absence of substrate (Doke et al., 1980). Therefore, in this study a more intermediate pH of 4.0 was chosen for both immobilization and first stage column elution.

Acidification of the 40-70 % fraction with concentrated buffer as previously reported by Kregar et al. (1977) followed by circulation of the supernatant through the column resulted in protein precipitation, which caused column clogging and reduced flow rates. Similar problems were encountered on circulating the 40-70 % ammonium sulfate fraction through a column pre-equilibrated with 0.1 M sodium acetate buffer of pH 4.0 containing 0.5 M sodium chloride. These problems were avoided by pump-circulating the sample in a column pre-equilibrated with 0.1 M sodium citrate buffer, pH 3.9, but without sodium chloride. Sodium acetate buffer was used only for elution of unbound protein.

General Performance and
Efficiency of the Glass
Bead-Pepstatin Column

The capacity of the glass bead-pepstatin column was estimated using bovine spleen cathepsin D. The total circulated, unbound, and immobilized activity were accounted for as shown in Table 1. There was a 9.5 % error on relating the total loaded activity and the sum of the unbound and eluted proteolytic activity. The results given in table 1 indicate that the total circulated activity (289.8 units) was far in excess of the column capacity. The column capacity is equivalent to the total number of units recovered in the pH 8.6 eluate following loading of sample containing excess enzyme. The capacity of the column was therefore 57.4 units corresponding to 19.8 % of the loaded activity (Table 1). The protein concentration in the concentrated pH eluate (2.2 ml in Table 1) was 0.6653 mg protein per ml and had a specific activity of 39.28 units per mg protein. Therefore the column had a capacity of immobilizing 1.4637 mg of cathepsin D.

The same column was used for purification of bovine spleen cathepsin D and bovine muscle cathepsin D and was run over 20 times without loss of capacity to immobilize the enzyme from the two tissue sources. Glass bead pepstatin columns therefore appear to be superior to AH-Sepharose 4B-pepstatin columns, with respect to reproducibility, resistance to pressure, durability and high porosity as has

Table 1. Determination of glass bead-pepstatin affinity column capacity for bovine spleen cathepsin D.

| FRACTION | TOTAL VOL (ml) | UNITS/ml | TOTAL |
|---|----------------|----------|-------|
| Total activity ^a (40-70 % AS) | 18.0 | 16.1 | 289.8 |
| Unbound activity ^b | 17.5 | 14.3 | 250.3 |
| pH 4.0 eluate ^c | 23.7 | 0.4 | 9.5 |
| pH 8.6 eluate ^d | 2.2 | 26.1 | 57.4 |

^a40 - 70 % ammonium sulfate fraction of bovine spleen, pH 4.0.

^bActivity remaining after circulation through affinity column for 45 minutes.

^cSome activity in column was not bound to pepstatin, and was removed in the pH 4.0 eluate.

^dActivity bound to pepstatin in the affinity column, and removed in the pH 8.6 eluate.

Total activity = 289.8 units.

Unbound activity + (pH 4.0 & pH 8.6 eluate activities) = 317.2 units.

previously been reported (Weetall, 1969; Robinson et al., 1971). Agarose based supports tend to compact on applying pressure and easily succumb to microbial degradation (Pormoraz and Meloan, 1978; Cuatrecasas and Anfinsen, 1971b; Guilford, 1973).

The Effect of pH on Cathepsin D Activity

The proteolytic activity of bovine muscle and spleen cathepsin D towards bovine hemoglobin was examined over the pH range 3.0 to 4.5. The results obtained are presented in Figures 3 and 4. The pH of highest activity for bovine spleen and muscle cathepsin D were pH 3.5 and 3.8, respectively.

There was little difference in proteolytic activity of bovine spleen cathepsin D at pH 3.2 (the pH used for bovine spleen cathepsin D assay throughout the study-Table 2) vs pH 3.5, the pH later determined to be optimum for bovine spleen cathepsin D (Figure 4). On the other hand, muscle cathepsin D had low activity at pH values below 3.5 (Figure 3). Similar pH differences between spleen cathepsin D and cathepsin D isolated from skeletal tissues have been reported. Whereas the optimum pH for bovine spleen cathepsin D activity has frequently been reported to be pH 3.5 (Anson, 1938; Press et al., 1960), the pH optimum of cathepsin D isolated from skeletal tissues has always been above pH 3.5. The pH 3.8 is optimal for cathepsin D isolated from chicken

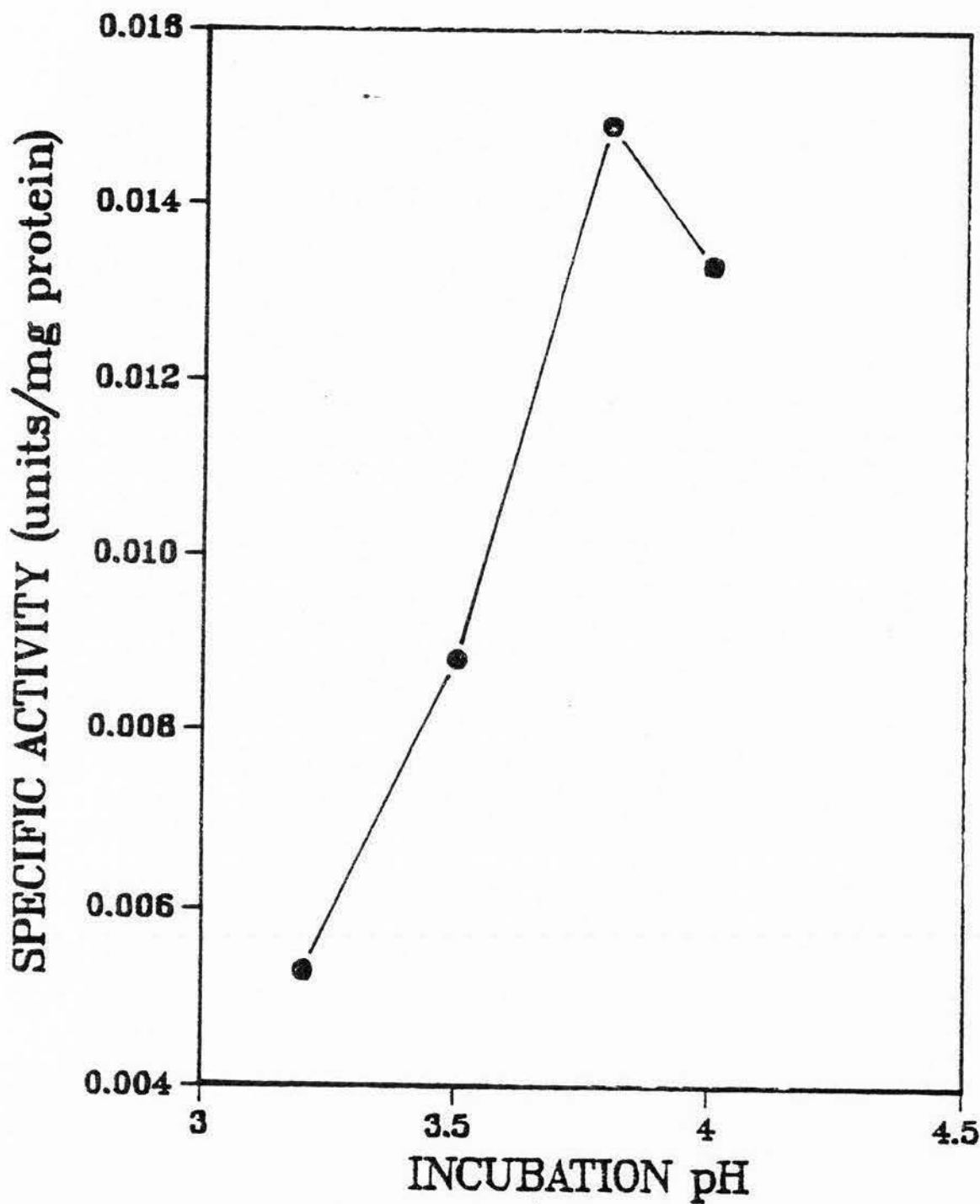


Figure 3. Effect of pH on bovine muscle cathepsin D proteolytic activity. The incubation mixture consisted of 0.5 ml of 2 % urea denatured hemoglobin, 1 ml of 0.2 M sodium formate buffer, and 0.5 ml of 40-70 % ammonium sulfate fraction. Incubation was at 37 C for 1 h.

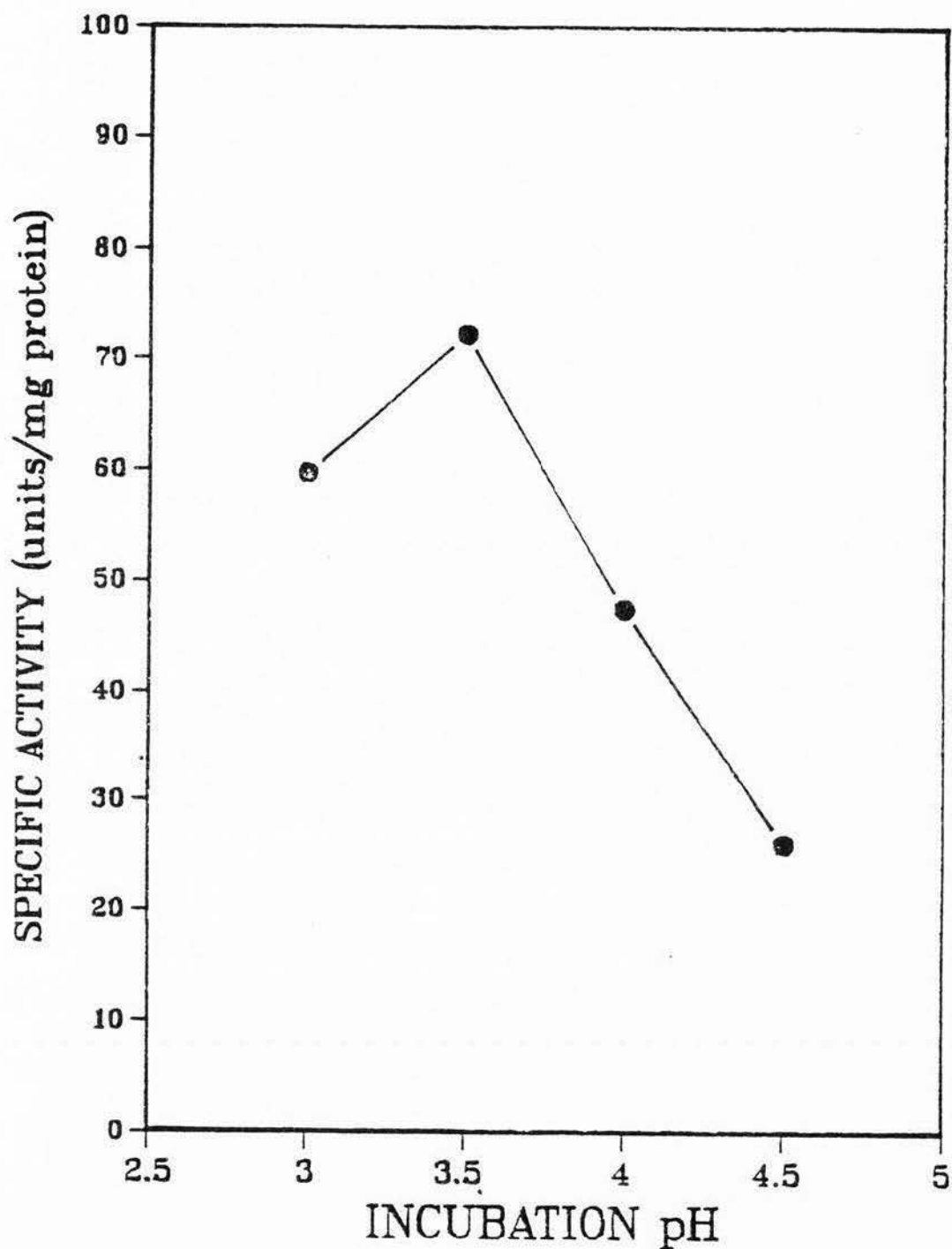


Figure 4. Effect of pH on bovine spleen cathepsin D proteolytic activity. A 2 ml incubation volume contained 1 ml of 0.2 M sodium formate buffer (of either pH 3.0, 3.5, 4.0 or 4.5), 0.5 ml of 2% urea denatured hemoglobin, 0.1 ml of water, and 0.4 ml of the pH 8.6 elutate (unconcentrated fraction).

Table 2. Purification table for bovine spleen cathepsin D by affinity chromatography using a glass bead pepstatin column.

| Fraction | mg protein per ml | Units ^a per ml | Total vol. (ml) | Total units | Units per mg protein | P.F1 ^b | P.F2 ^c % | Yield |
|---------------------------------|-------------------|---------------------------|-----------------|-------------|----------------------|-------------------|---------------------|-------|
| Crude homogenate | 48.1935 | 22.57 | 240.0 | 5416.8 | 0.468 | 1.0 | -- | 100.0 |
| Crude extract | 21.0464 | 22.62 | 151.0 | 3415.6 | 1.075 | 2.3 | 1.0 | 63.1 |
| 40% AS ^d supernatant | 10.6145 | 14.58 | 232.0 | 3382.6 | 1.374 | 2.9 | 1.3 | 62.5 |
| 40-70 % AS fraction | 23.4091 | 53.35 | 20.2 | 1077.7 | 2.279 | 4.9 | 2.1 | 19.9 |
| 40-70 % AS fraction, pH 4.0 | 5.5926 | 25.81 | 39.6 | 1022.1 | 4.615 | 9.9 | 4.3 | 18.9 |
| pH 8.6 eluate | 0.0453 | 3.27 | 24.2 | 79.1 | 72.200 | 154.3 | 67.2 | 1.5 |

^a1 unit is the amount of enzyme that releases 1 μ M L-tyrosine in 1 hr.

^bFold purification relative to crude homogenate.

^cFold purification relative to crude extract.

^dAmmonium sulfate.

muscle (Iodice et al., 1966), rat muscle (Bird et al., 1968), muscle of fresh water fish Tilapia mossambicus (Doke et al. (1980) and bovine muscle (Sliwinski et al., 1959; and Lutalo-Bosa and Macrae, 1969).

Hemoglobin dissolved in acid solution was the form of substrate preparation recommended for assaying bovine spleen cathepsins (Anson, 1938; Press et al., 1960). There is evidence that hemoglobin solutions in acid solutions prepared by the procedure of Anson (1938) cannot be successfully used for assaying cathepsin D from all types of tissues (Gianetto and de Duve, 1955; Sliwinski et al., 1959). The reason is largely due to the fact that cathepsin D isolated from different tissues displays activity towards hemoglobin over a pH range in which the extent of hemoglobin solubility is variable. The substrate prepared according to the procedure of Anson (1938) is suitable for assaying cathepsin D whose optimal pH for activity is below pH 3.5. On the other hand, such a substrate preparation is unsuitable for assaying cathepsin D whose optimal activity is above pH 3.8. Part of the reason is due to the low solubility of the substrate hemoglobin at a pH above 3.5 (Sliwinski et al., 1959; and Gianetto and de Duve (1955). Sliwinski et al. (1959) failed to obtain optimum bovine muscle cathepsin D activity on using bovine hemoglobin prepared according to the procedure of Anson (1938). Therefore, to assay cathepsin D over the pH range 3.0 to 4.5, urea denatured hemoglobin was used during pH-activity studies and

also for assaying bovine muscle cathepsin D at pH 3.8. Bovine spleen cathepsin D was routinely assayed at pH 3.2 using a 2 % hemoglobin solution in 0.1 M sodium citrate buffer, pH 2.8.

Effect of Time of Incubation on Bovine Cathepsin D Activity

The activity with incubation time of spleen cathepsin D purified 154.3 fold relative to the crude homogenate was approximately linear for the first 10 min of incubation at 37 C and pH 3.2 (Figure 5). To express all activity in similar units, i.e a unit of cathepsin D being 1 M L-tyrosine released per hour, the data for spleen cathepsin D was recalculated. This was necessary because spleen cathepsin D assays were conducted for 30 min, whereas the assays for muscle cathepsin D activity were conducted for 1 h to obtain sufficient color development. Since the bovine spleen cathepsin D time-activity curve was approximately linear through 10 min, the activity per hour was simply the activity per 10 min multiplied by 6.

Nonlinearity of the bovine spleen cathepsin D time-activity curve has been reported by Anson (1938) and Lutalo-Bosa and Macrae (1969). The time-activity curve (Figure 5) is approximately linear for the first 10 min of incubation, suggesting 10 min as an optimum incubation period for bovine spleen cathepsin D activity assays. A 10 min incubation period is consistent with results obtained in previous studies (Anson, 1938; Press et al., 1960). In one study, an

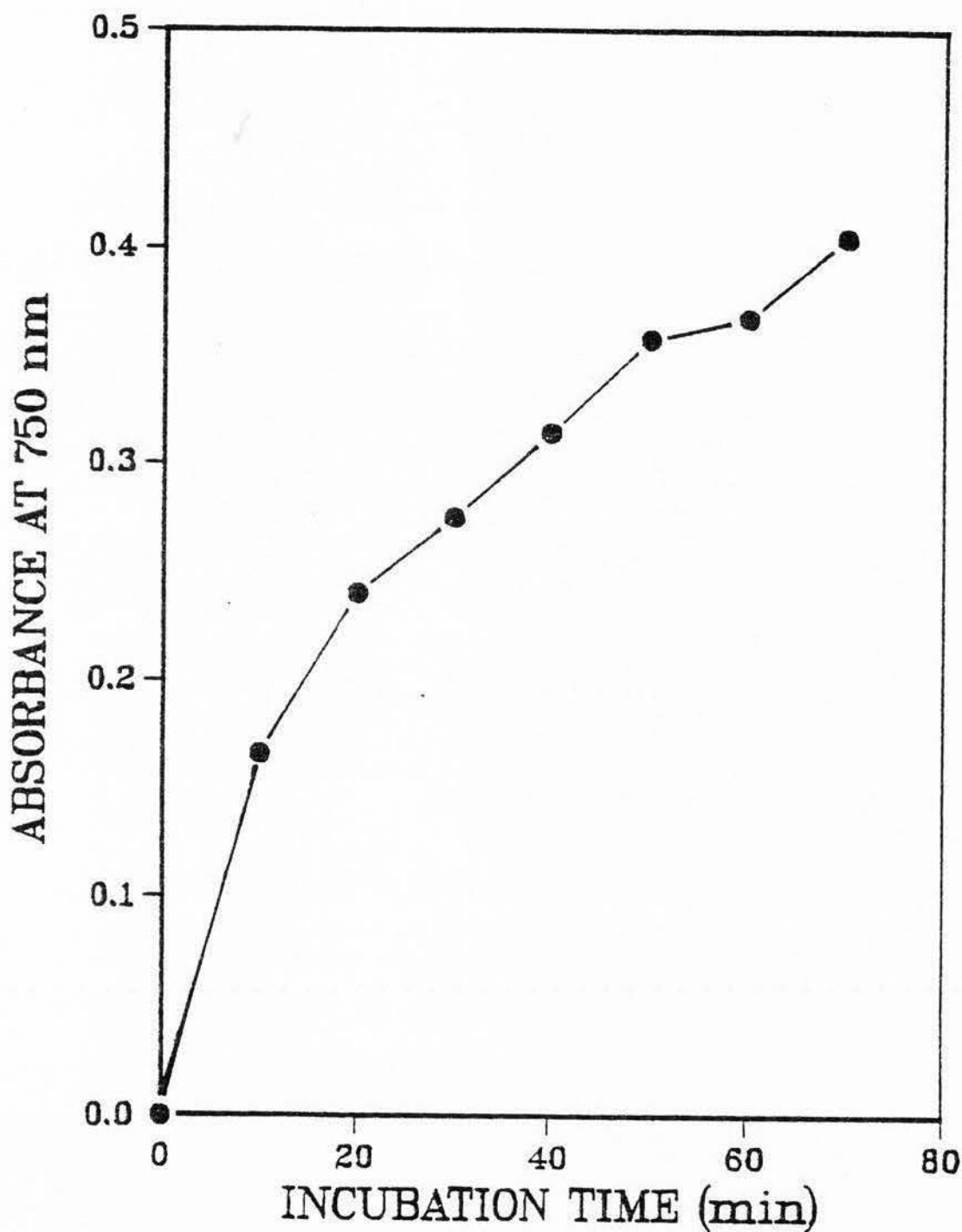


Figure 5. Effect of incubation time on the proteolytic activity of purified bovine spleen cathepsin D at pH 3.2. The concentrated enzyme purified by affinity chromatography (1.3 ml) was mixed with 24 ml of water and 26 ml of 2 % hemoglobin. Two milliliter samples were drawn at 10 minute intervals upto a maximum incubation period of 70 minutes.

optimum incubation period of 40 min was reported Yamamoto et al., 1979).

The time activity curve for bovine muscle cathepsin D was not determined in the present study. However, all muscle cathepsin D assay mixtures were incubated for 1 h as previously reported (Smith and Turk, 1974; Robbins et al., 1979; Kregar et al., 1977; Fan, 1981). According to Sliwinski et al. (1959) the time activity curve for bovine muscle cathepsin D is linear up to 4 h of incubation.

Characterization of Bovine Cathepsin D

Characterization of Purified Bovine Spleen Cathepsin D

Bovine spleen cathepsin D was purified 156.4 fold (relative to the crude homogenate) by affinity chromatography on a glass bead pepstatin column (Table 2). The yield relative to the crude homogenate was 1.5 %. SDS-polyacrylamide gels of the purified enzyme contained two prominent protein bands. A commercial preparation of spleen cathepsin D (Sigma Chemical Company, St. Louis, MO.) was also analysed by SDS-polyacrylamide gel electrophoresis. The electrophoretic pattern of the bovine spleen cathepsin D isolated by affinity chromatography (gel Y in Figure 6) was identical to that of the commercial preparation (gel Z in Figure 6). The two most prominent proteins in gels Y and Z (Figure 6) have molecular weights of about 45,000 and 35,000. On heavily loading the Sigma cathepsin D prepara-

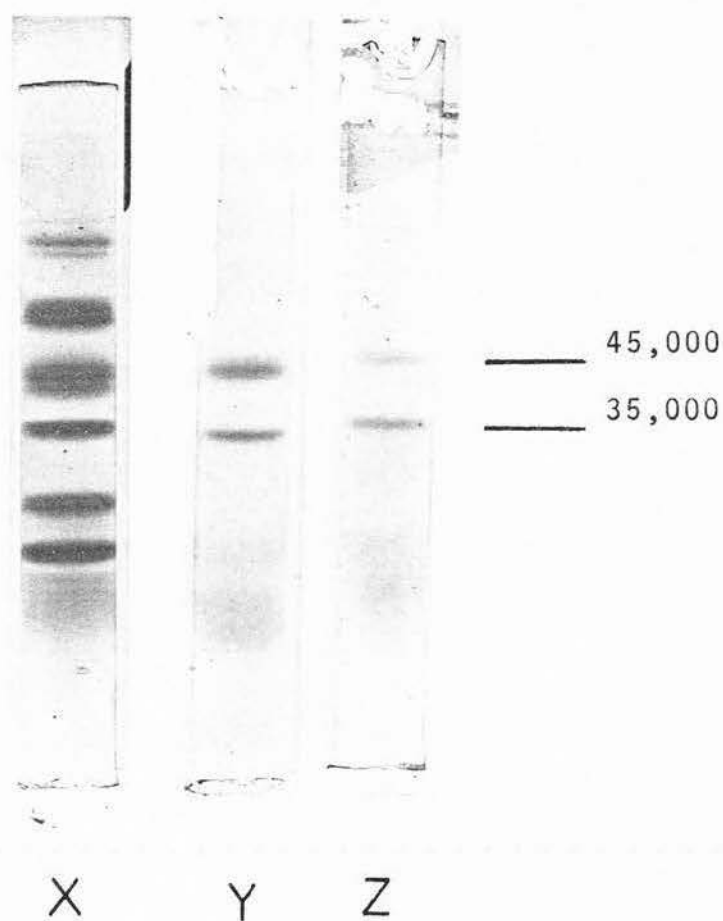


Figure 6. SDS gel-electrophoretic pattern of bovine spleen cathepsin D in 10 % gels at pH 8.6. The chamber buffer was 0.1 M Tris-glycine buffer containing 0.1 % SDS. Gel X represents low molecular weight standard proteins. Gel Y shows the pattern for a commercial preparation of bovine spleen cathepsin D. Gel Z is the pattern for bovine spleen cathepsin D purified by affinity chromatography on a glass bead-pepstatin column.

tion, there was evidence that it contained peptides with molecular weights of 17,000 to 20,000 daltons (Figure 7).

These findings confirm previous reports by Smith and Turk (1973) and Ferguson et al. (1973) that purified bovine spleen cathepsin D displays several protein bands when resolved by SDS-polyacrylamide gel electrophoresis. Smith and Turk (1974) reported an electrophoretic pattern of bovine spleen cathepsin D consisting of three prominent protein bands and a fourth band (which was of infrequent occurrence) which represented an unidentified high molecular weight peptide. SDS-polyacrylamide gel electrophoresis of bovine spleen cathepsin D revealed the presence of two major proteins having molecular weights of 42,000 and 28,000 (Ferguson et al., 1973).

Rat spleen cathepsin D does not display the molecular heterogeneity that is characteristic of bovine and porcine spleen. According to Yamamoto et al. (1979) a single band corresponding to 44,000 daltons was observed on SDS-polyacrylamide gels of purified rat spleen cathepsin D. Evidently, there is an interspecies diversity in the nature and molecular composition of cathepsin D isolated from spleen tissue. Bovine spleen cathepsin D is different from spleen cathepsin D isolated from either rat or mouse (Huang et al., 1979). Whereas rat and mouse cathepsin D contain monomeric and disulfide linked dimer forms (Yago and Bowers, 1975), porcine and bovine spleen cathepsin D exists in at least 4 monomeric forms (Huang et al., 1979). According to

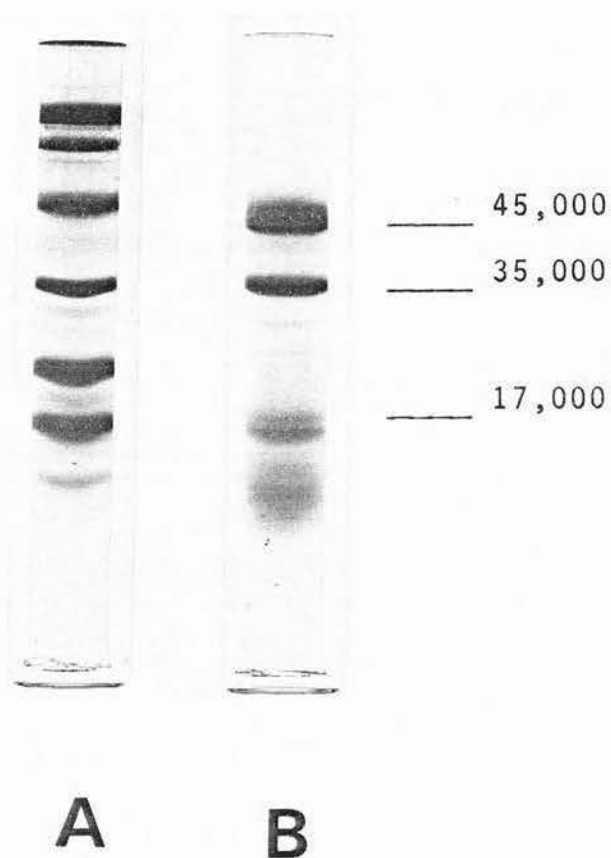


Figure 7. SDS-gel electrophoretic pattern of a commercial preparation of bovine spleen cathepsin D (Sigma Chemical Company, St. Louis, MO.). Procedures were as described for Figure 6.

Huang et al. (1979) the monomeric forms of porcine cathepsin D have molecular weights of 100,000, 50,000, 35,000, and 15,000 daltons. Bovine spleen had similar chain composition, but with minor differences in individual monomeric chains. Absence of dimeric forms of cathepsin D in bovine and porcine spleen was confirmed when treatment of a purified enzyme failed to produce lower molecular weight forms. It was theorized by Huang et al. (1979) that the 100,000 dalton unit is a precursor form which cleaves to form single chain peptides of about 50,000 daltons. The single chain form in turn produces two isozymes having molecular weights of 35,000 and 15,000.

The present study has confirmed the existence of the single chain form (MW=45,000) and a 35,000 dalton peptide. These peptides probably correspond to values of 50,000 and 35,000 daltons previously reported by Huang et al. (1979). However the 100,000 and 15,000 dalton components were not detected in the preparation of cathepsin D purified by affinity chromatography on a glass bead-pepstatin column. A peptide having a molecular weight close to 15,000 daltons (17,000 dalton band in gel B, Figure 7) was detected in commercial preparations on applying larger sample to a gel.

Characterization of Bovine
Muscle Cathepsin D Purified
by Affinity Chromatography
and by Ion Exchange/Gel
Filtration Chromatography

Bovine muscle cathepsin D was purified 24 fold using a glass bead-pepstatin column (Table 3). Like bovine spleen cathepsin D, the purified enzyme displayed molecular heterogeneity in having four proteins with molecular weights of 57,000, 45,000, 41,000 and 20,000 (Figure 8, gel A). The 57,000, 45,000, and 41,000 dalton components were the most dominant proteins. Thirty six hours after the first purification, the same 40-70 % ammonium sulfate fraction was purified by affinity chromatography column under similar experimental conditions. The peptide composition of the second run was analysed by SDS-polyacrylamide gel electrophoresis alongside the first preparation. The peptide composition for the second preparation are shown in Figure 8, gel B. The peptide composition in Figure 8, gel B shows that the 20,000 dalton component was the most predominant protein whereas the 45,000 and 41,000 dalton components present in the electrophoretogram of the first extract (Figure 8, gel A) were absent.

These results suggest that bovine muscle cathepsin D undergoes molecular autolysis with time. The 57,000 dalton component is probably the precursor form of muscle cathepsin D which undergoes spontaneous cleavage according to the scheme proposed in Figure 9. Cleavage at either site C or D

Table 3. Purification of bovine muscle cathepsin D by affinity chromatography.

| Fraction | mg protein per ml | Units ^a per ml | Total volume (ml) | Total units | Units per mg protein | P.F. ^b | Yield % |
|-------------------------|-------------------|---------------------------|-------------------|-------------|----------------------|-------------------|---------|
| Crude extract | 5.530 | 0.162 | 265.0 | 42.93 | 0.0293 | 1.0 | 100.0 |
| 40-70 % AS ^c | 24.050 | 0.800 | 20.8 | 16.64 | 0.0333 | 1.1 | 38.8 |
| pH 8.6 eluate | 0.085 | 0.060 | 11.0 | 0.66 | 0.7059 | 24.1 | 1.5 |

^aOne unit is the amount of enzyme that releases 1 μ M L-tyrosine per hour.

^bFold purification.

^cAmmonium sulfate.

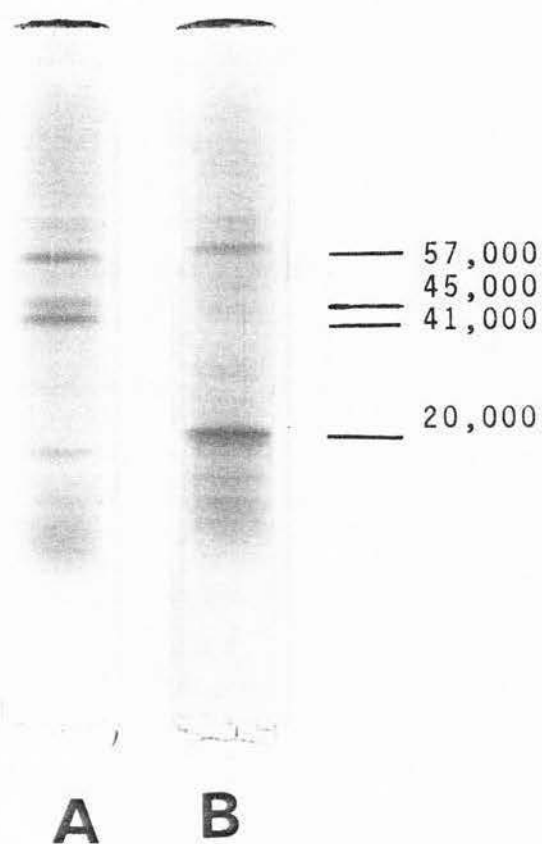


Figure 8. SDS-polyacrylamide gel electrophoresis of bovine muscle cathepsin D purified by affinity chromatography on a glass bead pepstatin column. Samples A and B were isolated 36 hours apart starting with the same stock of 40-70 % fraction. See materials and methods.

(Figure 9) would give peptides having molecular weight of 20,000, 45,000 and 41,000. Cleavage at site D alone would yield a 45,000 dalton component and a component having molecular weight in the region of 20,000 daltons. On the other hand, cleavage at site C will give two components, one having a molecular weight of 41,000 and the other with molecular weight between 20,000 and 30,000 daltons. Apparently the results shown in Figure 8, gel B suggest that both the 45,000 dalton and 41,000 dalton components are unstable over time and become cleaved at a site close to the center of the molecule (site B on the proposed degradation scheme in Figure 9). If the peptides having molecular weights of 40,000 to 45,000 undergo such a cleavage, there would be an increase in the amount of peptides having a molecular weight of about 20,000 as shown in Figure 8, gel B.

Although bovine muscle cathepsin D purified on a glass bead pepstatin column was of high purity as judged from the resolution pattern on SDS-polyacrylamide gels, its proteolytic activity towards urea-denatured bovine hemoglobin was low compared to that of bovine spleen cathepsin D purified under similar experimental conditions. The maximum specific activities of bovine spleen and muscle cathepsin D were 72.200 (Table 2) and 0.706 (Table 3) units per mg protein, respectively.

Bovine muscle cathepsin D from the 40-70 % ammonium sulfate fraction was further purified by ion exchange chro-

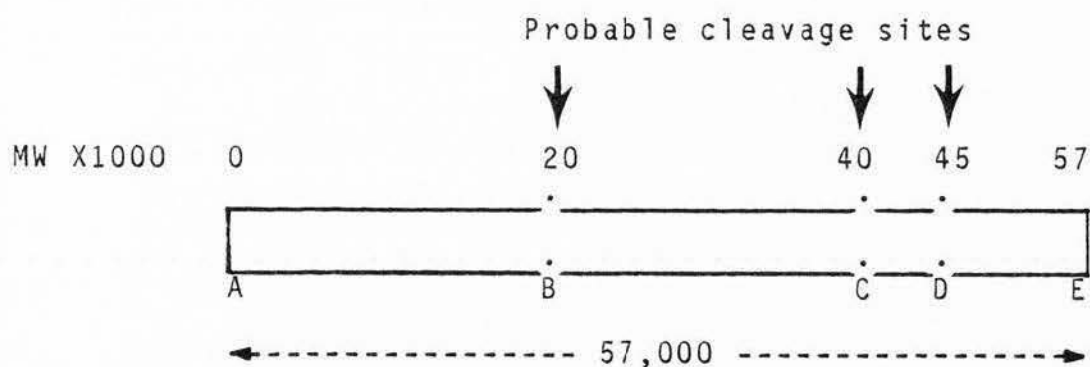


Figure 9. A schematic presentation of the probable mechanism by which native bovine muscle cathepsin D undergoes autodegradation. B, C, and D are probable cleavage sites, with cleavage at sites C and D occurring almost immediately after onset of rigor.

matography on DEAE-sephadex A-50 (Figure 10). This trial was done as an attempt to obtain purified cathepsin D by conventional chromatographic methods and for comparison with cathepsin D preparations obtained by affinity chromatography. The second peak from the ion exchange column (eluted by 0.1 M sodium phosphate buffer, pH 7.0 containing 0.14 M NaCl) was applied to a Sephadex G-100 gel filtration column (Figure 11). A 17-fold purification relative to the crude extract was obtained following gel filtration (Table 4). The specific activity of muscle cathepsin D purified by conventional techniques (ion exchange, gel filtration) was similar to that of enzyme purified by affinity chromatography. The specific activity of the enzyme purified by the conventional techniques was 0.778 units per mg protein (Table 4). The specific activity of the enzyme purified by affinity chromatography was 0.708 units per mg of protein (Table 3).

SDS-polyacrylamide gel electrophoresis of the enzyme fraction obtained from gel filtration on Sephadex G-100 showed 3 major proteins having molecular weights of 63,000, 45,000, and 41,000 (Figure 12). Similar results have been reported by Okitani et al. (1981). According to Okitani et al. (1981), attempts to further purify the fraction by repetition of gel filtration resulted in a loss of enzyme activity. The most remarkable outcome of the purification of muscle cathepsin D by affinity chromatography versus conventional chromatographic procedures is the similarity of the respective electrophoretograms, shown in Figures 8 and 12.

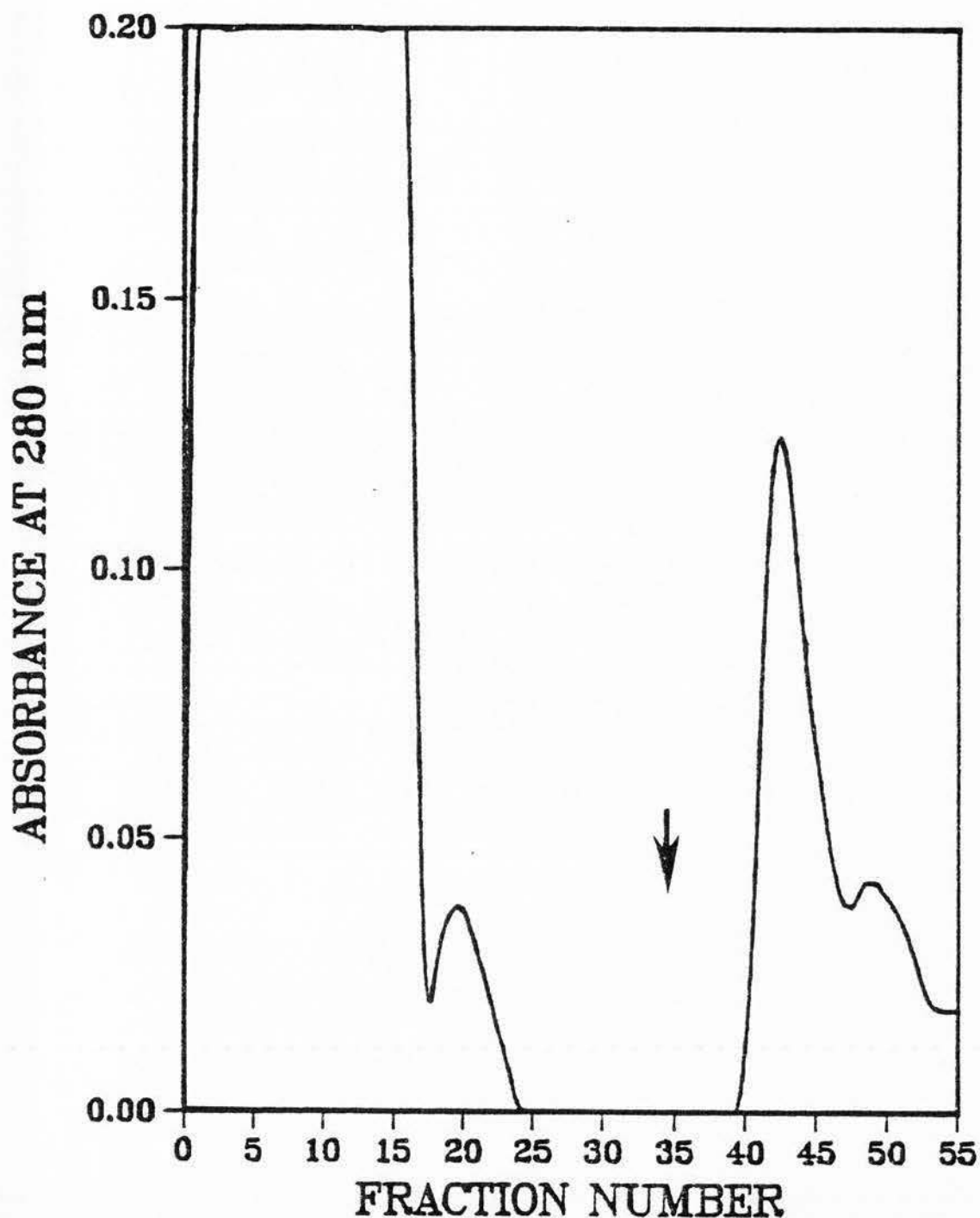


Figure 10. Elution pattern of bovine muscle cathepsin D on DEAE-Sephadex A-50 (1.0 X 50 cm column). Three milliliter fractions were collected at a flow rate of 15 ml per hour. Fractions 1-24 were eluted with the equilibration buffer (0.1 M sodium phosphate buffer, pH 7.0). The fractions containing cathepsin D were eluted with 0.1 M sodium phosphate buffer containing 0.14 M sodium chloride (see arrow). Fractions 41-50 were pooled and concentrated to 5 ml, half of which was further purified by gel filtration.

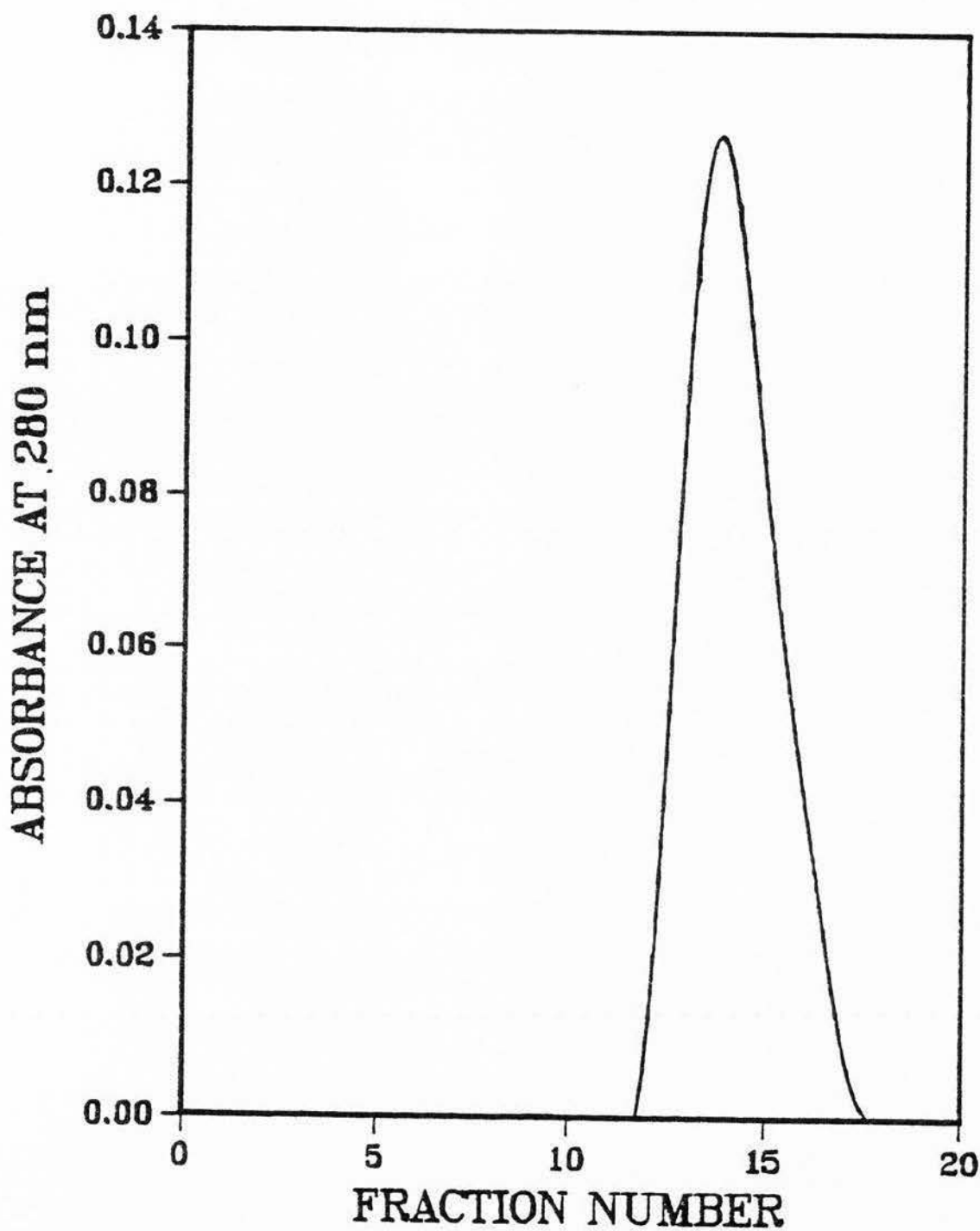


Figure 11. Elution pattern of concentrated DEAE-Sepahadex A-50 fractions from bovine muscle on a Sephadex G-100 column (1.0 X 25 cm column). The column was equilibrated and eluted with 0.010 M sodium phosphate buffer, pH 6.0. Fractions of 2 ml were collected at a flow rate of 4 ml per hour.

Table 4. Purification of bovine muscle cathepsin D by ion exchange chromatography using DEAE-Sephadex A-50 followed by gel filtration using Sephadex G-100.

| Fraction | mg protein per ml | Units ^a per ml | Total ml | Total units | Units per mg protein | P.F. ^b | Yield % |
|-------------------------------------|-------------------|---------------------------|----------|-------------|----------------------|-------------------|---------|
| Crude extract | 9.28 | 0.425 | 265.0 | 112.625 | 0.046 | 1.0 | 100.0 |
| 40-70 % AS ^c supernatant | 4.01 | 0.221 | 432.0 | 95.472 | 0.055 | 1.2 | 84.8 |
| 40-70% AS fraction | 37.60 | 2.759 | 18.5 | 51.000 | 0.073 | 1.6 | 45.3 |
| DEAE-Sephadex A-50 | 0.19 | 0.086 | 22.9 | 1.969 | 0.453 | 9.8 | 1.8 |
| Sephadex G-100 | 0.09 | 0.070 | 27.5 | 1.925 | 0.778 | 17.0 | 1.7 |

^aOne unit is defined as the amount of enzyme that releases 1 μ M L-tyrosine per hour.

^bFold purification.

^cAmmonium sulfate.

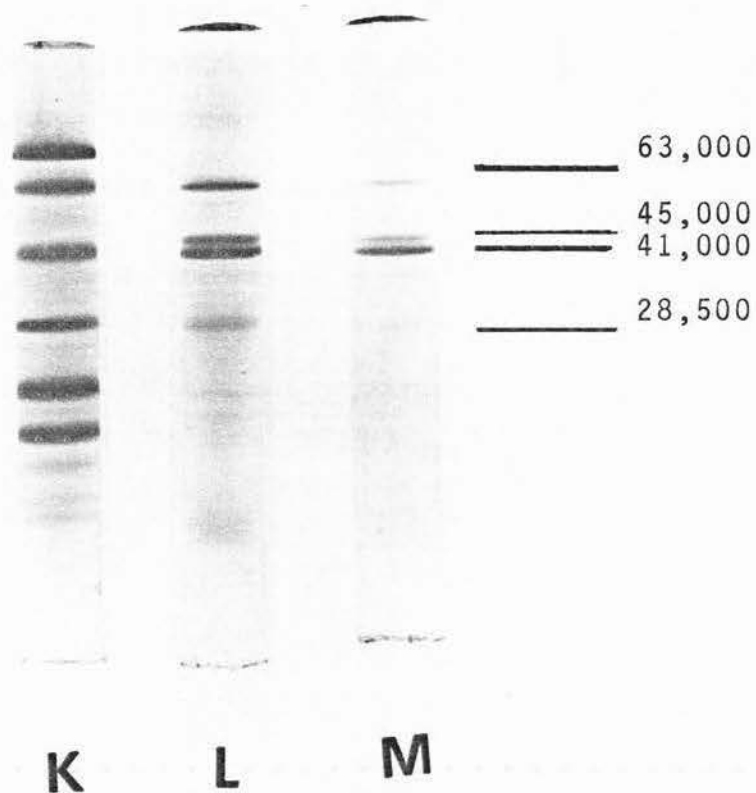


Figure 12. SDS-polyacrylamide gel electrophoresis of bovine muscle cathepsin D fractions eluted from DEAE-Sephadex A-50 (Gel L) and further purified by gel filtration on Sephadex G-100 (Gel M). Gel K represents the low molecular weight protein standard.

Conventional chromatographic procedures have recently been reported to be better than affinity chromatographic techniques for purification of cathepin D from skeletal tissues (Okitani et al., 1981), due to low activity and yield achieved on adopting the affinity purification techniques. Okitani et al. (1981) attributed reduced specific activity and enzyme yield with affinity chromatography to non-specific binding of impurities. Contrary to these observations, use of glass beads as a support material in the present study did not cause non-specific binding during purification of bovine muscle cathepsin D. Evidence for this is based on the high degree of column reproducibility in terms of consistency of the SDS-gel pattern of fractions from each run.

Bovine muscle cathepsin D had 0.98 to 1.08 % of the proteolytic activity of bovine spleen cathepsin D, if the the specific activities of muscle cathepsin D (Tables 3 and 4) are expressed as percentages of the specific activity of bovine spleen cathepsin D (Table 2). According to Lutalo-Bosa and Macrae (1969) the specific activity of bovine muscle cathepsin D was 25 % of the specific activity of bovine spleen cathepsin D isolated from rat liver. Bouma and Gruber (1964) reported the specific activity of cathepsin D isolated from rat skeletal muscle to be 7 % of that of liver cathepsin D. The specific activity of chicken breast muscle cathepsin D was reported to be 5 % of that of bovine spleen cathepsin D (Iodice et al., 1966). Low proteolytic action

of muscle cathepsin D appears to be a universal feature for all mammalian species.

Additional evidence consistent with an inherent difference between bovine muscle and spleen cathepsin D are differences in optimum times for incubation at 37 C for detectable proteolytic action towards bovine hemoglobin. Due to the relatively high proteolytic activity of bovine spleen cathepsin D towards hemoglobin, short incubation periods ranging from 10 min (Press et al., 1960; Anson, 1938) to 40 min (Yamamoto et al., 1979) have been reported. On the other hand, the minimum recommended incubation period for bovine muscle cathepsin D is 1 h (Robbins and Cohen, 1976; Robbins et al., 1979; and Okitani et al., 1981). Incubation periods of up to 4 h have also been reported for muscle cathepsin D activity assays (Sliwinski et al., 1959). To date there is no valid explanation for the low proteolytic action of muscle cathepsin D towards hemoglobin, which by convention has been used for assaying the activity of cathepsin D from all tissues. Differences in molecular weight, subunit composition and optimal pH for proteolytic activity towards hemoglobin, have been demonstrated in the present study between muscle and spleen cathepsin D. The term "cathepsin D" was coined by Press et al. (1960) in reference to the specific bovine spleen lysosomal acid protease which had no proteolytic action towards the synthetic substrates of cathepsins A, B, and C. The term has since then been extended to all enzymes isolated from other tissue as long as such isolates

had some proteolytic action towards bovine hemoglobin at pH below 4 and had a molecular weight in the range of 30,000 to 60,000 daltons.

Nature of Isolated Intramuscular Connective Tissue

The intramuscular connective tissue isolated from bovine shank muscle had an average of 61.3 % collagen on a dry weight basis (Table 5). This percentage lies within the range previously reported for collagen in bovine intramuscular connective tissue. The % collagen in connective tissue is related to the tissue source, age and sex of the animal. The mean % collagen in lyophilized connective tissue residues isolated from biceps femoris muscle of veal, steers, cows and aged cows are 52.1 ± 7.3 , 70.2 ± 7.8 , 72.3 ± 3.9 , 67.0 ± 6.4 respectively (Goll et al., 1963). Acid soluble collagen was extracted from the connective tissue extract and analysed by SDS-polyacrylamide gel electrophoresis in 7.5 % gels. The electrophoretic pattern of the acid soluble collagen are illustrated in Figure 13, gel C. Gel B represents the pellet from which acid soluble collagen had been extracted. The results shown in Figure 13 suggest that most of the connective tissue isolated was insoluble due to the obvious small amount of α -chains extracted as shown in gel C (Figure 13). This is what can be expected for connective tissue extracted from aged animals, whose intramuscular collagen has large amounts of intermolecular crosslinks and

Table 5. Percentage collagen in intramuscular connective tissue (IMCT) isolated from beef shank muscle.

| SAMPLE | WEIGHT | HYDROXY- PROLINE | COLLAGEN * EQUIVALENTS | TOTAL AS % OF IMCT |
|--------|--------|---------------------|---------------------------|--------------------------|
| No. | mg | mg | mg | |
| I | 20.0. | 1.63 | 12.2 | 61.0 |
| II | 20.0 | 1.65 | 12.3 | 61.5 |
| Mean | - | 1.64 | 12.2 | 61.3 |

* Collagen =hydroxyproline X 7.46 (Woessner and Brewer, 1963).

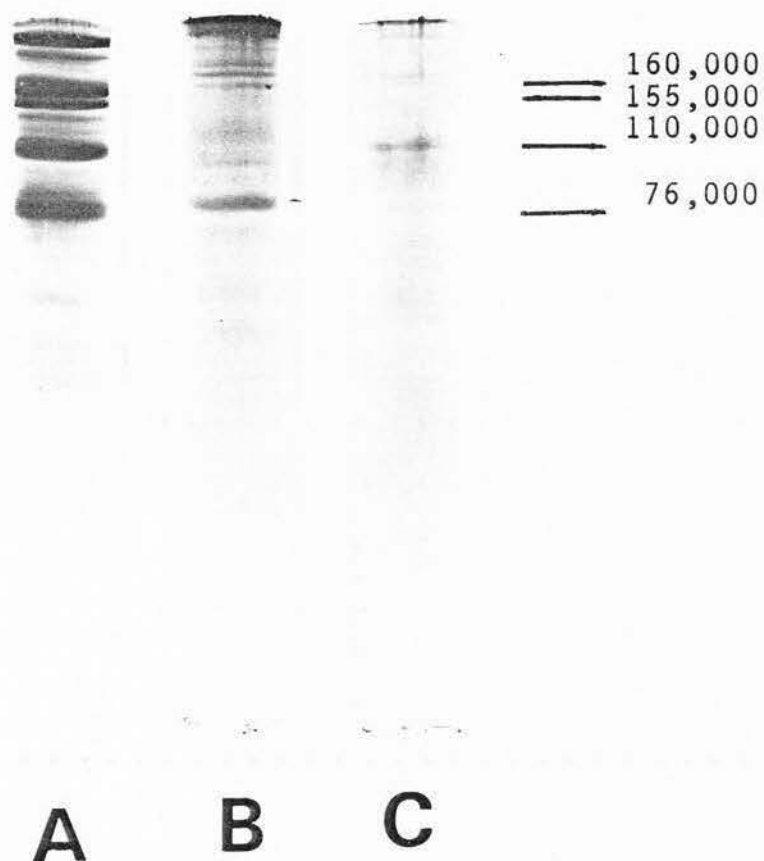


Figure 13. SDS-polyacrylamide gel electrophoresis of intramuscular connective tissue in 7.5 % gels. The electrophoresis was run in 0.1 M sodium phosphate buffer, pH 8.0 containing 0.1 % SDS. Gel A shows the high molecular weight protein standard. Gels B and C shows the pattern for insoluble and acid soluble collagen respectively.

thus is of low extractability (Goll et al., 1963). The connective tissue was extracted from a commercial grade animal and therefore of advanced age.

Action of Cathepsin D and Bromelain on
Intramuscular Connective Tissue

Cathepsin D extracts from bovine muscle and spleen had no digestive action on intramuscular connective tissue over the pH range 3.0 to 4.5 following incubation for 24 h at 37 C (data not shown). Failure to release free hydroxyproline, or soluble collagen from the intramuscular connective tissue was confirmed by a direct test for free hydroxyproline in the digest filtrates and HCl hydrolysate of the filtrates. Similar observations were made on incubating the intramuscular connective tissue with a commercial preparation of bovine spleen cathepsin D (Sigma Chemical Company, St. Louis, MO.).

Tests for free hydroxyproline in the filtrates from intramuscular connective tissue digests by pineapple stem bromelain for all assay mixtures incubated over the pH range 3.0-4.5 were negative. These findings were conclusive that bromelain was not digesting intramuscular connective tissue to amino acids. However, presence of hydroxyproline containing peptides in the filtrate was confirmed following acid hydrolysis of the intramuscular connective tissue digests. The collagen equivalents for the released hydroxyproline were computed using a factor of 7.46 (Woessner and Brewer,

1963) giving the estimates of collagen released following incubation of IMCT with pineapple stem bromelain over the pH range 3.0-4.5 (Figure 14, APPENDIX D, Table 9). In samples of IMCT incubated with bromelain, over 50 % of the soluble collagen was released during the first 8 h of incubation (Figure 14).

The digestion of intramuscular connective tissue by bromelain was pH dependent. The amount of released collagen peptides increased with incubation time and decreased with increasing pH as illustrated in Figure 14. Evidently, pineapple stem bromelain has a component capable of digesting insoluble connective tissue under low pH conditions. This is in agreement with reports by Minami et al. (1971) and Yoshimoto et al. (1974). According to Beddows et al. (1976) and Beddows and Ardeshir (1979) pineapple stem bromelain efficiently converted insoluble nitrogen to soluble nitrogen during production of fermented fish sauce. A study by Yoshimoto et al. (1974) was conclusive that plant-derived enzymes such as bromelain are more active in digesting connective tissue fractions of beef under acidic conditions. Similarly pineapple stem bromelain has been reported to contain components having proteolytic activity under acid conditions (Minami et al., 1971)

SDS-polyacrylamide gel electrophoresis of the intramuscular connective tissue digests following incubation with pineapple stem bromelain at pH 3.0 indicated that two peptides with molecular weights of about 62,000 and 44,000

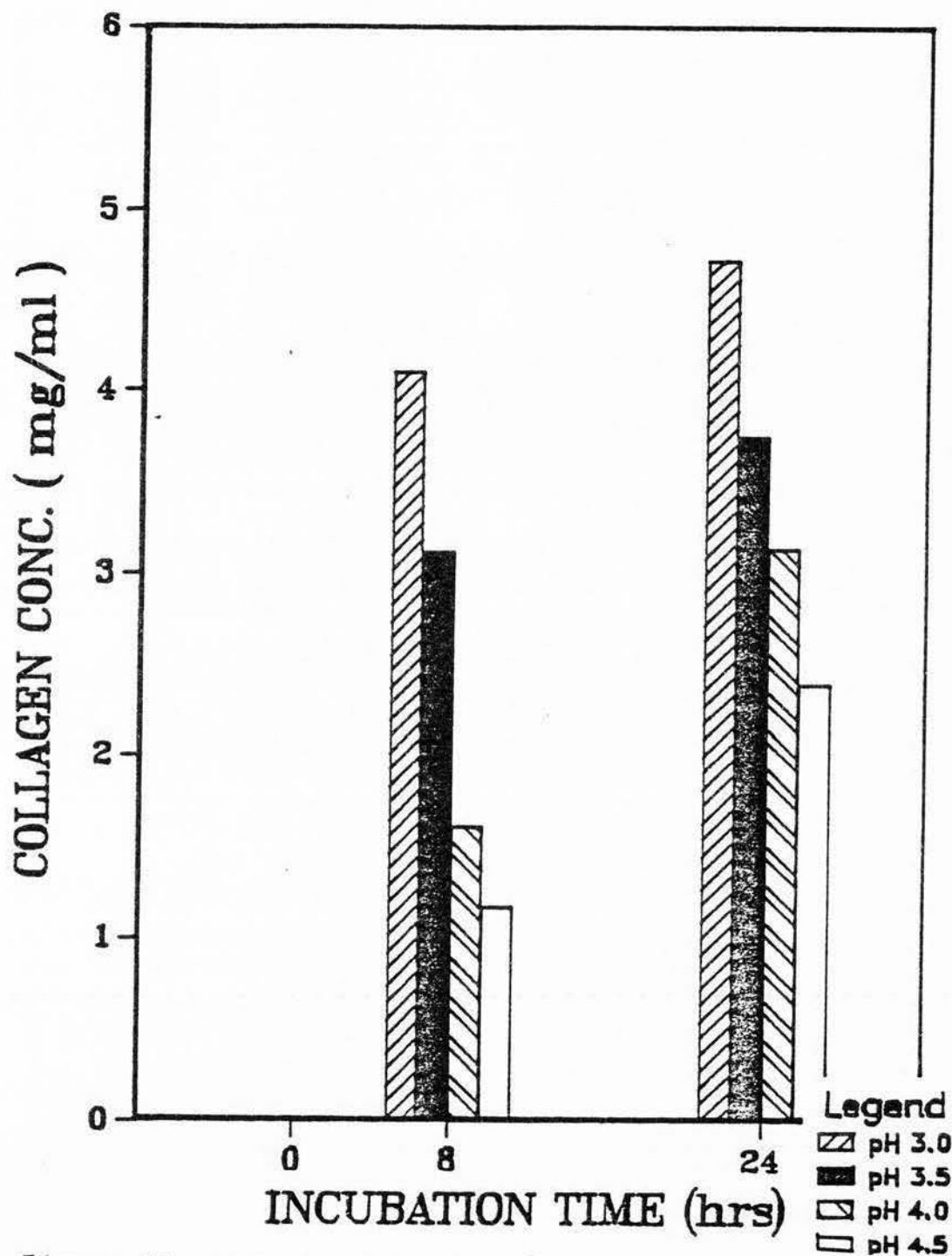


Figure 14. Effect of incubation time and pH on the extent of release of soluble connective tissue from samples of intramuscular connective tissue incubated with pineapple stem bromelain. Collagen values were obtained by multiplying hydroxyproline values by a factor of 7.46 (Woessner and Brewer, 1963).

daltons were released, as shown in gels G and H (Figure 15), as compared to controls (gels D and E) without enzyme treatment. These two peptides had a tendency to increase with incubation time. Only those peptides appearing in the enzyme treated samples and not appearing in the two controls (i.e. intramuscular connective tissue incubated with and without enzyme, D & E in figure 15 respectively) were identified as products of enzyme hydrolysis. Both controls had a high molecular weight peptide (MW=115,000) which was also present in the enzyme treated sample drawn within the first 30 min of incubation (Figure 15, gel F). One of its hydrolytic products is probably a peptide with a molecular weight of 71,000 which was present in trace amounts in the controls D and E and yet appears as a distinct band in enzyme treated samples drawn after 8 (Gel G) and 24 (Gel H) hours of incubation.

A close examination of Figure 15 shows that the enzyme also digested the high molecular weight proteins which appear in the control gels D and E. These high molecular weight proteins gradually disappeared with increase in incubation time as shown in gels F, G, and H (Figure 15). Since the test for hydroxyproline following acid hydrolysis of the control filtrates were negative, these high molecular weight proteins were not collagen, but probably some of the myofibrillar proteins which were present as minor contaminants in the isolated intramuscular connective tissue. According to Beddows et al. (1976) and Beddows and Ardeshir (1979)

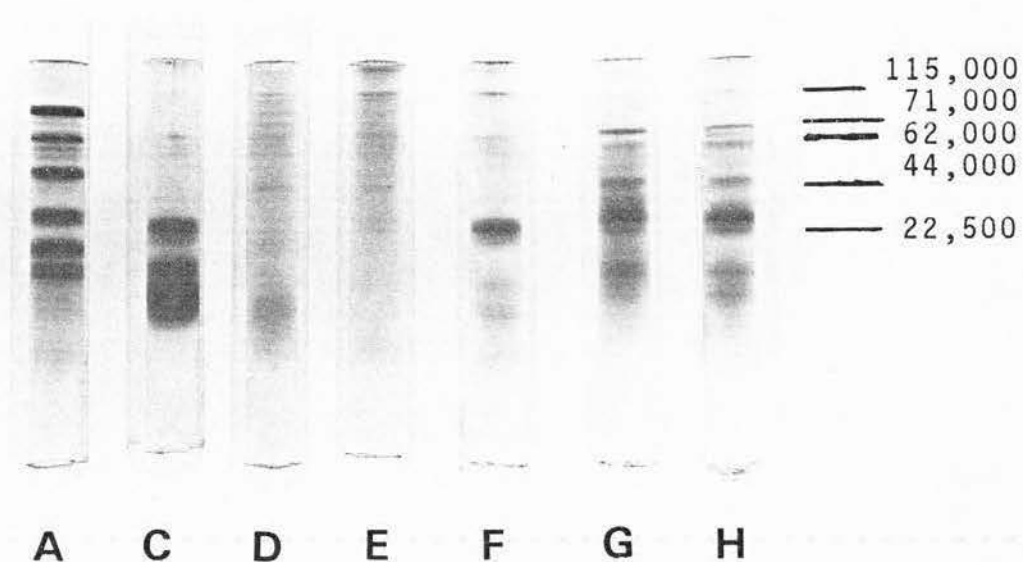


Figure 15. Electrophoresis of intramuscular connective tissue digest filtrates following incubation with pineapple stem bromelain at pH 3.0. Gel A represents Low molecular weight protein standard proteins. Gel C shows the pattern for pineapple stem bromelain. D and E are controls with inactivated enzyme and without enzyme respectively. Gels F, G, H shows the patterns obtain for samples drawn at 0, 8 and 24 h incubation at pH 3.0. Controls D and E had been incubated at pH 3.0 for 24 h.

bromelain digests insoluble collagen as well as myofibrillar proteins. The optimal pH for digestion of myofibrillar proteins by bromelain is pH 6-7 (Yang et al., 1975).

To release hydroxyproline-containing peptides bromelain may act upon the intramolecular cross-links of IMCT leading to release of α -chains. Alternatively, bromelain may attack the α -chains on either side of the cross links, resulting in release of tropocollagen fragments. The chains were further cleaved into two peptides of molecular weights 44,000 and 62,000 daltons. The sum of the molecular weights of the two peptides gives a molecular weight of 108,000 daltons, which lies in the molecular weight range for collagen α -chains. According to Veis (1964) the molecular weight of α -chains of tropocollagen lies between 80,000 and 125,000 daltons. Apparently, the total collagen solubilized following acid hydrolysis of connective tissue digest after 8 and 24 h of incubation (Figure 14) were similar and this is consistent with the minor difference in intensity of protein bands obtained after 8 and 24 h of incubation (gel G and F in Figure 15).

Since cathepsin D isolated from bovine muscle and spleen tissue had no action on intramuscular connective tissue it can be inferred that cathepsin D is not capable of cleaving collagen cross links. The intermolecular crosslinks in collagen are not peptide bonds (Eyre, 1980), but rather aldol condensation Schiff base bonds between adjacent side chains, predominantly in the short telepeptide region at

either end of an α -chain. Cathepsin D may have a slight action on released connective alpha chains in the presence of a second enzyme with ability to cleave tropocollagen inter and intramolecular cross-links, thereby releasing or solubilizing the collagen. According to Dingle et al. (1972), Kazakova and Orekhovich (1972), Ferguson et al. (1973) and Brostoff et al. (1974) cathepsin D preferably acts upon a Phe-Phe bond. A slight action has been reported on peptide bonds having Phenylalanine and a few other aromatic and aliphatic amino acids (Whitaker and Seyer, 1979b). The probability of having the Phe-Phe bond or other preferred peptide bond combination within the collagen molecule is quite small due to the low content of phenylalanine and tyrosine in collagen. Phenylalanine and tyrosine in bovine intramuscular collagen represent only 1.35 and 0.35 % of the amino acid residues respectively (McClain et al., 1971). With this low content of phenylalanine in collagen, the probability of having either a Phe-Phe or Phe-Tyr in collagen is quite small. Accurate information on the exact amino acid sequence in native collagen is not available, due to the presence of large amounts of glycine, proline and hydroxyproline in collagen and the tendency for repetitive sequences of the form Gly-X-Y (Veis, 1970). Sequence studies on cyanogen bromide collagen fragments has been reported (McClain et al., 1971). Such sequences do not detail the exact order of the amino acids in the native α -chains. Consequently, evidence is

non existent indicating the presence of Phe-Phe or Phe-Tyr bonds in collagen which will render collagen susceptible to the proteolytic action by cathepsin D. On the other hand the ability for cathepsin D to degrade myofibrillar proteins is well documented. According to Fan (1981), and Robbins et al. (1979) cathepsin D has a digestive action on myofibrils causing a release of a 30,000 dalton component.

The connective tissue cross links apparently are preferably acted upon by sulfhydryl proteases. Cathepsin B1 has been reported to digest connective tissue (Etherington, 1976; Burleigh et al., 1974). Kang and Rice (1970) reported that the meat proteins acted upon by bromelain were the insoluble proteins and myofibrillar proteins. Bromelain is known to be a sulfhydryl enzyme (Kang et al., 1974; Yang et al., 1975; Liener, 1973). A lysosomal enzyme that is thiol-dependent and has been reported to have digestive action on collagen is cathepsin B (Barrett, 1970; Etherington, 1976; Burleigh et al., 1974). On the other hand cathepsin D which is non-thiol dependent (Huang and Tappel, 1972; Turk and Kregar, 1978) has no proteolytic action on collagen as has been demonstrated in the present study and in previous studies (de Fremery and Streeter, 1969; Winstanley, 1979). It is therefore probable that connective tissue is digested by sulfhydryl enzymes only.

Under in vivo conditions cathepsin D could be involved in connective tissue turnover under conditions which have not yet been simulated in vitro. The argument that cathe-

psin D could act on connective tissue is largely based on studies involving its activity in involuting uterine tissue postpartum which is manifested with a concomitant increase in serum and urinary hydroxyproline. Such observations led to the general believe that the release of hydroxyproline was due to the action of cathepin D which is also reported to have high activity in such tissues. Woessner (1962) attributed the release of free hydroxyproline to prolidase and prolinase or a different protease having a pH optima at pH 3.5. Woessner (1962) speculated that the uterine protease was a specific collagenase which may be different from catheptic enzymes occurring in either muscle or spleen.

In a subsequent study by Woessner (1965) it was concluded that the uterine protease was not a true collagenase, since on incubating the uterine homogenate with collagen at pH 3 to 4 there was digestion, but on being incubated at neutral pH, no digestion was observed even in the presence of stabilizers and activators like calcium, EDTA and thiols. It was thus concluded that the uterine collagenolytic enzyme was a "general enzyme" that will attack collagen only in the pH range of 3.0 to 4.0. It was further theorized that the digestive vacuoles to which the lysosomes empty their enzymes have an acid environment favorable for acid digestion.

Anderson (1969) tested the digestive action of a liver granular fraction on collagen over the pH range 3 to 4 (sodium formate buffers) and observed that maximal collage-

nolytic activity was taking place at pH 3.45. Indications were that the fraction had some enzyme which Anderson (1969) referred to as "some form of cathepsin D". According to Ledward et al. (1975) meat collagen is relatively stable postmortem although recent work has indicated that a small but significant breakdown does occur during storage (Dutson and Lawrie, 1974; Wu et al., 1981). β -galactosidase, hyaluronidase and collagenase are the key enzymes responsible for connective tissue breakdown during ageing of meat (Wu et al., 1981). The β -galactosidases and hyaluronidase act on the ground substance of connective tissue to cause release of galactose and hyaluronic acid residues from acid mucopolysaccharides of proteoglycans without any direct effect on the collagen molecules.

CONCLUSION

Cathepsin D from muscle and spleen display differences with respect to peptide composition, proteolytic activity towards hemoglobin, optimum pH for proteolytic activity, and stability of the native form of the enzyme over time. Such differences lead to a general conclusion that the term "cathepsin D" does not refer to a unique enzyme, but to a highly diversified group of lysosomal enzymes. Therefore, in making reference to a cathepsin D preparation, it is emphasized that the species and tissue source must be specified.

Hemoglobin has been used as a substrate for assaying skeletal cathepsin D on the assumption that it has proteolytic activity comparable to that of cathepsin D extracted from either spleen or liver. However, skeletal muscle cathepsin D had low activity towards this substrate. Therefore, hemoglobin should not be used as a universal substrate for assaying cathepsin D from all tissue sources due to substrate preference among the cathepsin D group of lysosomal enzymes. Future attempts to study the proteolytic activity of muscle cathepsin D should therefore first establish the most ideal substrate for its assay.

The results of the present study have been conclusive that cathepsin D from either muscle or spleen has no proteolytic action towards intramuscular collagen. This conclusion is based on *in vitro* incubation studies using a highly

purified cathepsin D preparation. In previous studies, crude preparations of lysosomal fractions which had cathepsin D and other enzymes were used and the results from such studies have given rise to speculation that cathepsin D has proteolytic action on connective tissue. Incubation of crude lysosomal extracts containing cathepsin D and traces of cathepsin B will result in some connective tissue proteolysis, as the latter enzyme has been reported to have proteolytic activity towards connective tissue. Pineapple stem bromelain was used in the current study for comparison purposes, and had substantial proteolytic activity towards intramuscular connective tissue. Since cathepsin B and pineapple stem bromelain are both sulfhydryl enzymes, it is probable that connective tissue is hydrolysed by a sulfhydryl group of lysosomal enzymes. The meat tenderizing action of cathepsin D is therefore not due to its proteolytic action towards connective tissue but due to its reported proteolytic action towards myofibrillar proteins.

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APPENDICES

Appendix A

Table 6. Preparation of the L-tyrosine standard curve.

| Final Tyr. conc $\mu\text{M}/\text{ml}$ | μL of stock solution 100 ml ^a | Tyrosine solution (ml) | 0.1 M NaOH (ml) | Phenol reagent (ml) | A_{750} ^b |
|---|---|------------------------|-----------------|---------------------|------------------------|
| 0.000 | 0.0 | 0.5 | 1.0 | 0.3 | 0.000 |
| 0.002 | 72.0 | 0.5 | 1.0 | 0.3 | 0.034 |
| 0.005 | 180.0 | 0.5 | 1.0 | 0.3 | 0.083 |
| 0.010 | 360.0 | 0.5 | 1.0 | 0.3 | 0.146 |
| 0.015 | 540.0 | 0.5 | 1.0 | 0.3 | 0.208 |

^a100 ml = 100,000 μL (and the corresponding dilution factors are obtained by dividing 100,000 by the microliters of stock solution used). The concentration of stock solution was 10 μM L-tyrosine/ml.

^bEach absorbance value is an average of 4 measurements.

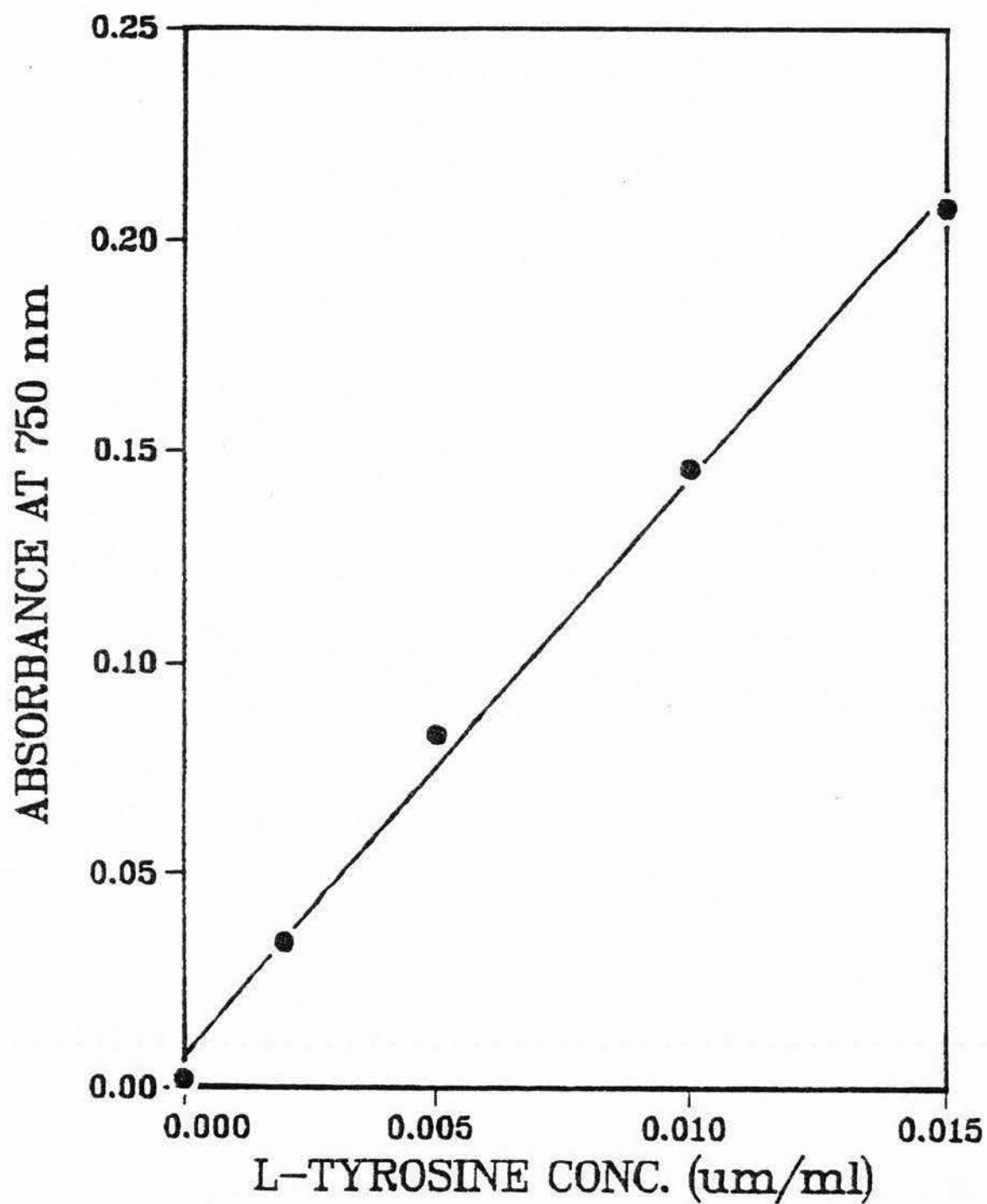


Figure 16. L-tyrosine standard curve.

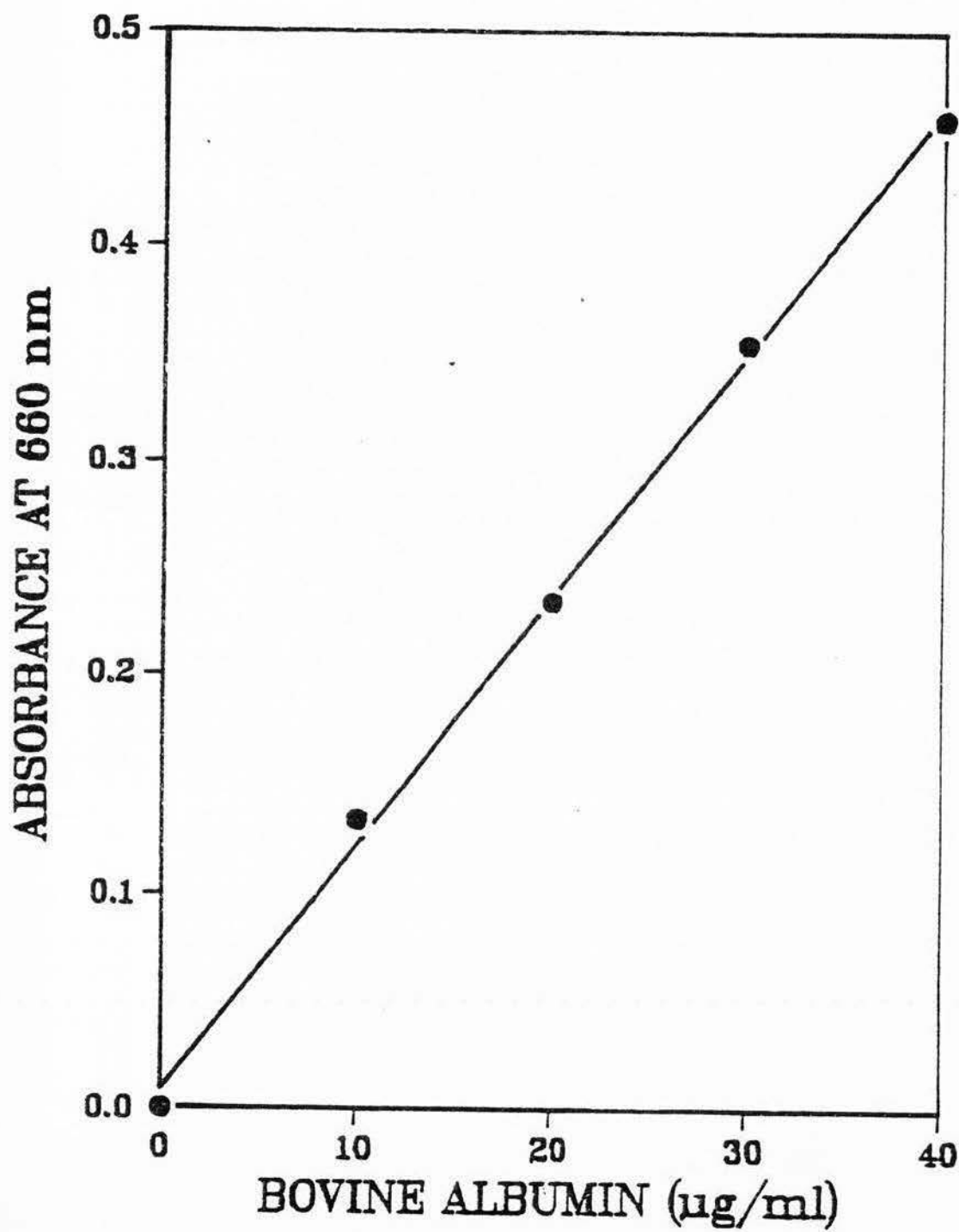
Appendix B

Figure 17. Protein standard curve.

Appendix C

Table 7. The relative mobility of low molecular weight standard proteins in 7.5 and 10 % sodium dodecyl sulfate (SDS)-polyacrylamide gels.

| PROTEIN | MOLECULAR WEIGHT | AVERAGE Rf IN 7.5 % GELS (pH 8.0) | AVERAGE Rf IN 10 % GELS (pH 8.5) |
|--------------------|------------------|---|--|
| Phosphorylase b | 92,500 | 0.16 | 0.26 |
| B.S.A | 66,200 | 0.25 | 0.35 |
| Ovalbumin | 45,000 | 0.37 | 0.48 |
| Carbonic anhydrase | 31,000 | 0.52 | 0.59 |
| S. T.I. | 21,500 | 0.61 | 0.72 |
| Lysozyme | 14,400 | 0.68 | 0.81 |

B.S.A= Bovine serum albumin
S.T.I= Soybean trypsin inhibitor

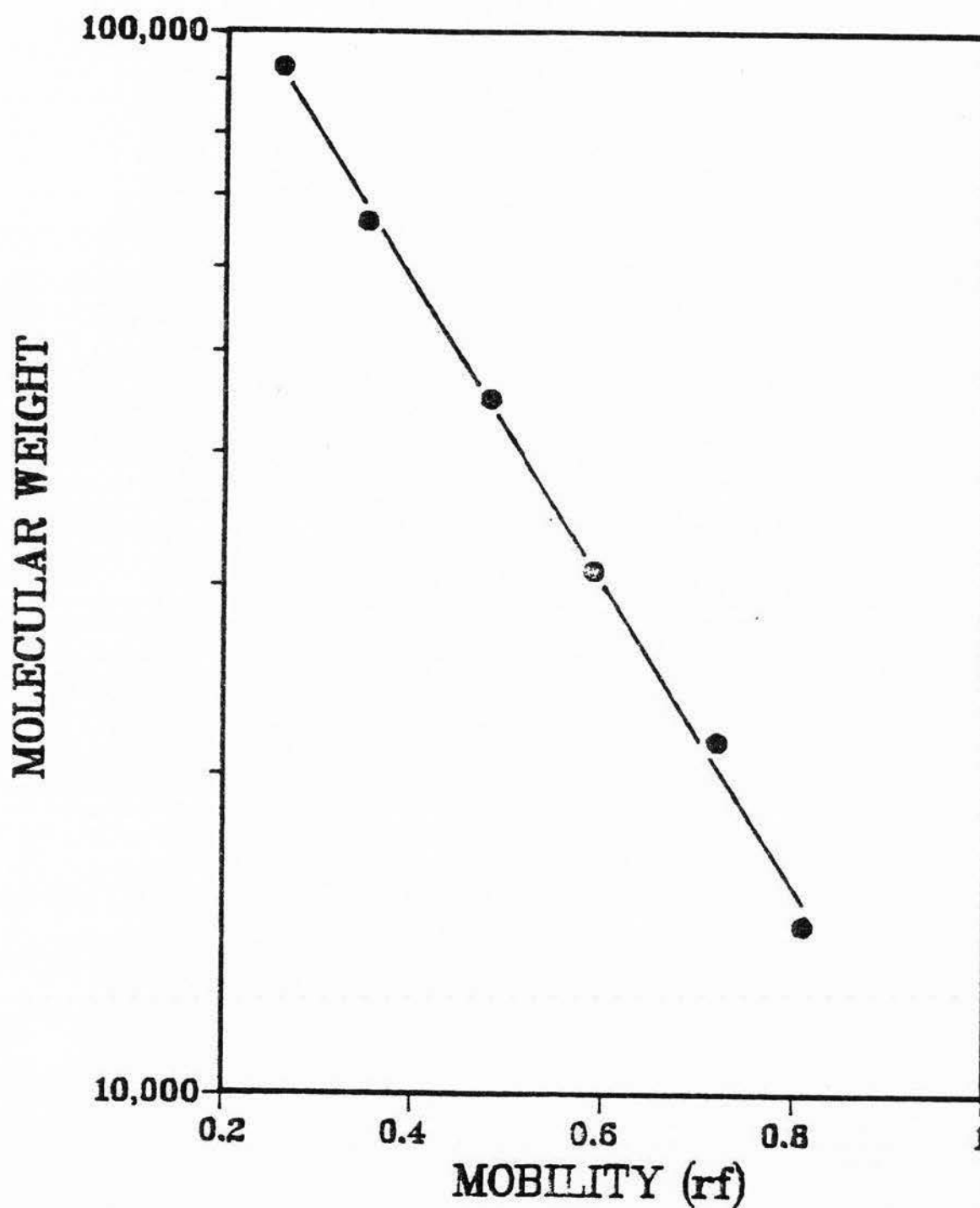


Figure 18. Standard plot of molecular weight vs mobility for low molecular weight protein standards in 10 % polyacrylamide gels. The molecular weight of the protein standards is given in appendix C, table 7.

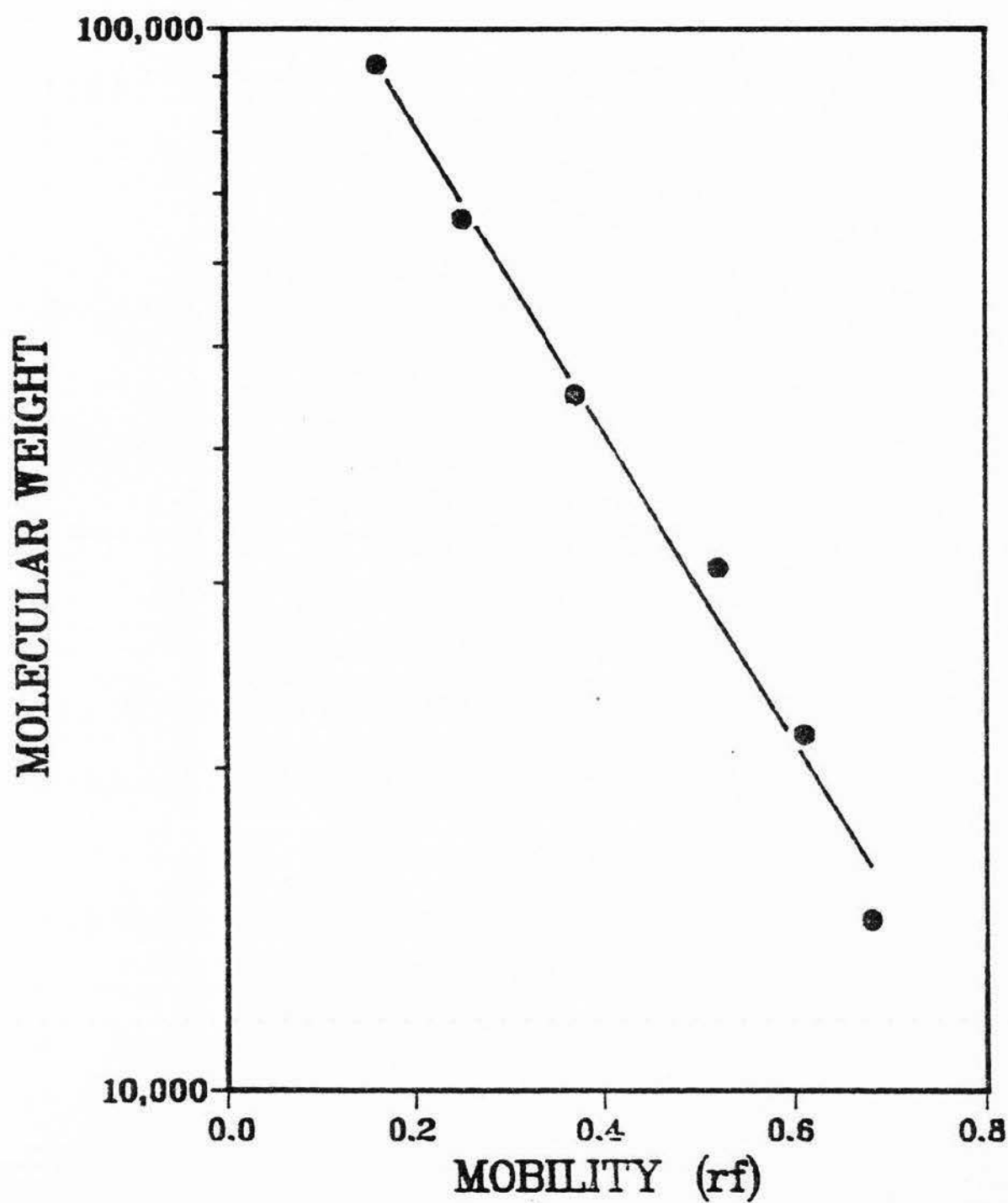


Figure 19. Standard plot of molecular weight vs mobility for low molecular weight protein standards in 7.5 % gels. The molecular weight of the protein standards is given in appendix C, table 7.

Table 8. The relative mobilities of high molecular weight proteins after SDS-polyacrylamide gel electrophoresis using 7.5 % gels at pH 8.0 (0.1 M sodium phosphate buffer).

| PROTEIN | MOLECULAR WEIGHT | AVERAGE Rf |
|-----------------|------------------|------------|
| Myosin | 200,000 | 0.03 |
| B.G. | 116,250 | 0.19 |
| Phosphorylase b | 92,500 | 0.26 |
| B.S.A | 66,200 | 0.37 |
| Ovalbumin | 45,000 | 0.52 |

B.G.=Beta galactasidase
B.S.A=Bovine serum albumin

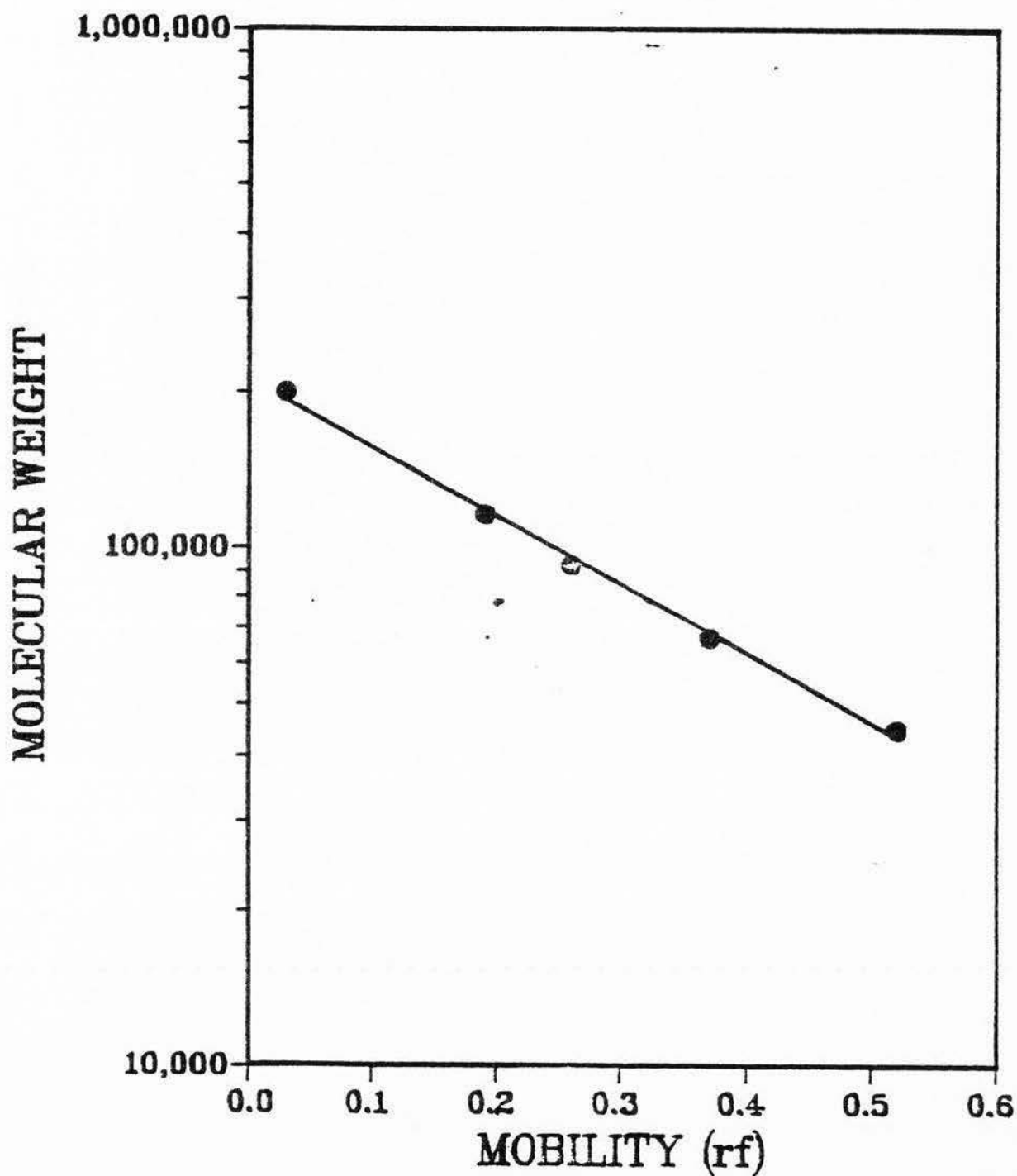


Figure 20. Standard plot of molecular weight vs mobility for high molecular weight protein standards in 7.5% gels. The molecular weight of the protein standards is given in appendix C, table 8.

Appendix D

Table 9. Solubilization of collagen in intramucular connective tissue by pineapple stem bromelain.

| Time (hours) | INCUBATION pH | | | |
|------------------------------|---------------|------|------|------|
| | 3.0 | 3.5 | 4.0 | 4.5 |
| Solubilized collagen (mg/ml) | | | | |
| 0 | 0.00 | 0.00 | 0.00 | 0.00 |
| 8 | 4.10 | 3.11 | 1.61 | 1.17 |
| 24 | 4.72 | 3.75 | 3.14 | 2.40 |

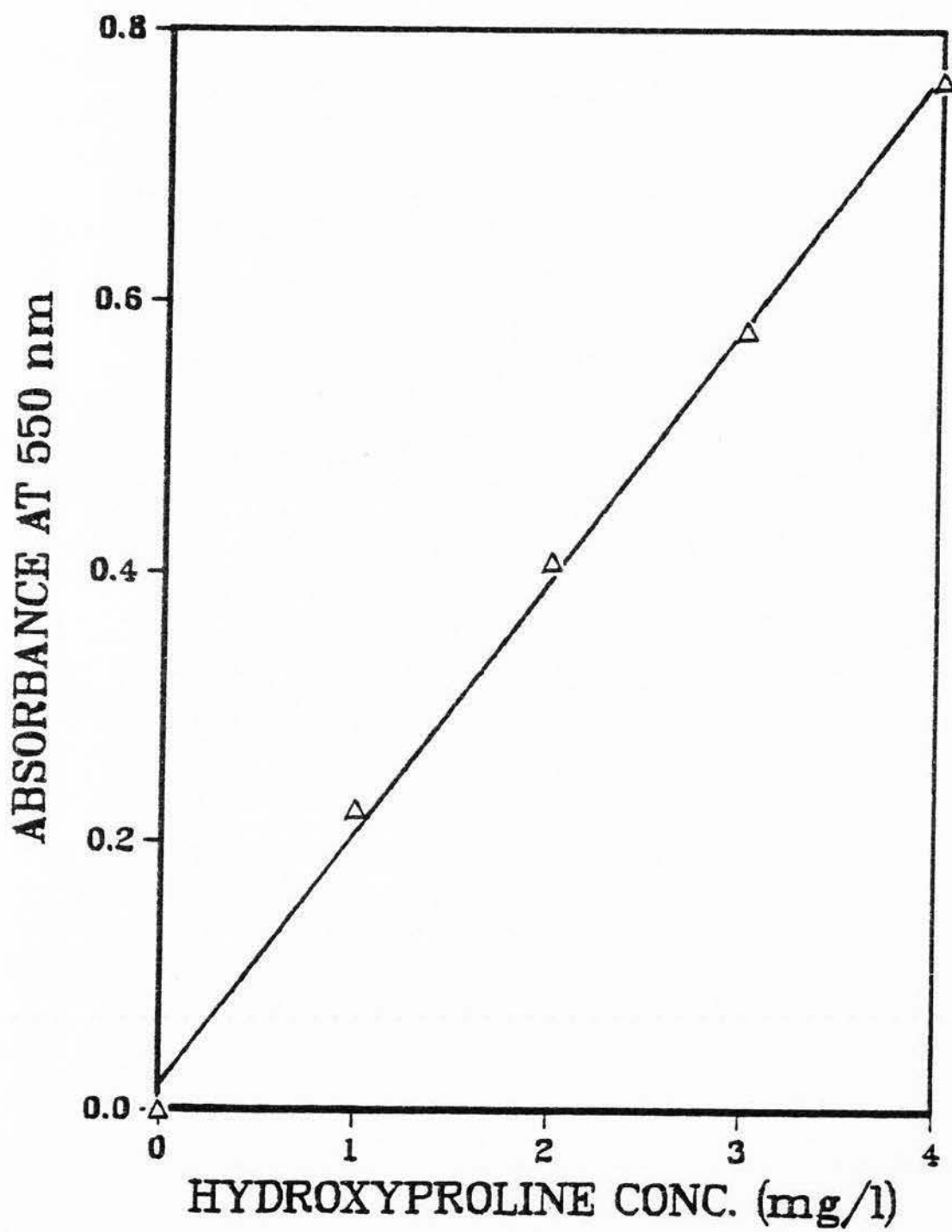


Figure 21. Hydroxyproline standard curve.

Appendix E

Table 10. A summary of the major features which distinguish the cathepsins. The letters Y and N mean "yes" and "no" respectively. The symbol - indicates that no test was conducted.

| Feature | CATHEPSIN | | | | | | REFERENCE |
|------------------------------------|-----------|---|---|---|---|---|--------------------------|
| | A | B | C | D | E | S | |
| Hemoglobin Digestion | Y | Y | Y | Y | - | - | Press et al. (1960) |
| | | | | | Y | | Lapresle and Webb (1962) |
| | | | | | | Y | Turk and Kregar (1978) |
| Cleaving B-chain of insulin | N | N | N | Y | - | - | Press et al. (1960) |
| | | | | | Y | - | Turk and Kregar (1978) |
| | | | | | | Y | Barrett (1970) |
| Inhibition by Pepstatin | N | N | N | Y | Y | - | Smith and Turk (1974) |
| | | | | | | Y | Turk and Kregar (1978) |
| Inhibition by thio-reactive agents | N | Y | Y | N | Y | - | Yago and Bowers (1975) |
| | | | | | | Y | Turk and Kregar (1978) |

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