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A HIGH MOLECULAR WEIGHT SULFHYDRYL DEPENDENT PROTEINASE FROM BOVINE SPLEEN

By

Thomas J. Farrell

A Thesis Submitted to the Faculty of the Graduate School of
Loyola University of Chicago in Partial Fulfillment
of the Requirements for the Degree of
Master of Science

May

1987

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CHAPTER I

INTRODUCTION

It is generally accepted that in eukaryotes the lysosome is an important site of degradation of proteins and other constituents of the organism. This belief is supported by the presence of a variety of hydrolases in these organelles (Dean and Barrett, 1976). Formation of lysosomes containing digestive enzymes and substrates is preceded by two events. First, lysosomal acid hydrolase proteins are synthesized by the rough endoplasmic reticulum and then packaged within small particles or vesicules through an interaction with the Golgi region (Gregoriadis, 1975). This collection of acid hydrolases compartmented away from the rest of the cytoplasm by a bounding membrane is termed a primary lysosome. Second, infoldings of the cell membrane produce vacuoles containing extracellular material. These endocytic vacuoles and primary lysosomes can then fuse and form a secondary lysosome containing endocytosed material which becomes a substrate for the lysosomal hydrolases (De Duve and Wattiaux, 1966). Subsequent degradation of proteins within the lysosome results in the formation of free amino acids and small peptides (Tappel, 1969).

Under certain conditions a volume of cytoplasm containing mitochondria, endoplasmic reticulum and other cytoplasmic constituents may become enclosed by an outer limiting membrane. This autophagic vacuole may fuse with primary lysosomes to yield a secondary lysosomal structure (Koenig, 1969). Mortimore and Mondon (1970) provided evidence that, apart from the role of autophagocytosis in the disposal of injured cytoplasmic components, the lysosome functions in the continuous process of intracellular protein turnover. Proteolysis was found to occur only in incubations of whole organ homogenates and not in particle-free supernatants. The particulate fraction capable of releasing acid soluble material from prelabeled endogenous proteins was identified as lysosomal by the comigration of the products of endogenous proteolysis with lysosomal enzymes in density equilibrium centrifugation (Neely and Mortimore, 1974).

The earliest evidence for the existence of the lysosome as a separate subcellular particle was based on the distribution and structure-linked latency of five acid hydrolases including acid phosphatase and acid protease (De Duve, _et al_, 1955). A separation procedure based on differential centrifugation was then developed to obtain subcellular fractions enriched in acid hydrolases (Applemans, _et al_, 1955). Subsequent observations by De Duve

(1955) demonstrated that cathepsin (acid protease) showed a similar distribution pattern to acid phosphatase, suggesting that these enzymes were located within the same class of cytoplasmic granules, which were termed lysosomes. Various lines of evidence now indicate a heterogeneity of lysosomes in size, density and in the kinds and number of hydrolases contained (Beck and Lloyd, 1969).

Much evidence further indicates a multiplicity of pathways for protein degradation. The degradation of normal cellular proteins of limited size, but of exceptionally high turnover rate, is probably nonlysosomal in nature, since degradation of these classes is not much influenced by nutritional deprivation (Poole and Wibo, 1973) or by inhibitors of lysosomal proteases (Knowles and Ballard, 1976). While strong evidence exists indicating that basal degradation of longer-lived proteins is insensitive to agents that inhibit lysosomal proteolysis (Libby and Goldberg, 1981), lysosomotropic agents such as chloroquine have been found to inhibit protein breakdown that has been enhanced by nutritional deprivation (Poole, et al, 1978).

The requirement of intracellular protein breakdown for a supply of metabolic energy was first described by Simpson (1953) who observed that release of amino acids from labeled proteins in rat liver slices is inhibited by anaerobic conditions or by inhibitors of cellular energy

production. Hershko and Tomkins (1971) used nutritional deprivation to show that inhibitors of protein synthesis block only the 'enhancement' of protein breakdown, while inhibitors of energy production also suppress basal protein degradation. The initial events of autophagy may involve processes of membrane assembly or rearrangement which may well require energy. In addition, energy may be required for the maintenance of intralysosomal pH necessary for the action of acidic proteases.

This acidic pH optimum for catalysis is an important property of many lysosomal hydrolases. Histochemical studies suggest that the contents of lysosomes are acidic (Dean and Barrett, 1976) and biochemical studies have provided support for an ATP dependent proton pump in the lysosomal membrane which may be partially responsible for an acidic intralysosomal pH (Schneider, 1979). At an acidic pH, a rapid hydrolysis of acid denatured proteins is expected. Numerous studies have yielded evidence for the existence of a large number of enzymes capable of hydrolyzing peptide bonds. Further, a synergistic mode of action has been proposed for these peptide hydrolases (De Duve and Wattiaux, 1966) which may be divided into exopeptidases which cleave bonds only near the ends of polypeptide chains, and endopeptidases which cleave bonds away from the ends. The activity of exopeptidases is such that it is difficult to detect partially degraded protein molecules in

cells. Once endopeptidases have opened the way for the exopeptidases, extensive digestion takes place rapidly.

The acid protease origionally named 'cathepsin' (Wilstatter and Bamann, 1929) is now named Cathepsin D, and the term cathepsin is now a general name for enzymes referred to as tissue proteinases. The earliest cathepsins to be described included Cathepsins A-E (Barrett, 1969), a heterogeneous group of peptide hydrolases all with a pH optimum in the acid range. The current convention is to retain the name of cathepsin for the endopeptidases, but to name the exopeptidases in a semisystematic manner that is partially based on their specificity. Thus, five classes of exopeptidases are recognized: These comprise the Aminopeptidases and Carboxypeptidases, which cleave single amino acid residues from N and C termini respectively; the Dipeptidylpeptide hydrolases and Peptidyldipeptide hydrolases, which cleave dipeptides from N and C termini; and dipeptidases which split dipeptides. Accordingly, for example, Cathepsin A is now more commonly known as Lysosomal Carboxypeptidase A and Cathepsin C is known as Dipeptidyl Peptidase I.

No satisfactory scheme has been produced for the classification and naming of endopeptidases on the basis of their specificity, and since systematic names cannot be constructed, trivial names such as Cathepsin are used. Recently described endopeptidases, named as Cathepsins,

include Cathepsins F (Dingle, et al, 1977), G (Plow, 1980), H (Schwartz and Barrett, 1980), J and K (Liao and Lenney, 1984), L (Kirschke and Barrett, 1982), M (Pontremoli, et al, 1982), N (Etherington, 1972), R (Langner, et al, 1982), S (Turnsek, et al, 1975) and T (Hargrove, et al, 1982). A useful classification system has been based on the recognition of four distinct classes of catalytic mechanisms (Hartley, 1960), and it has been proposed that the tissue proteinases be classified as carboxyl, thiol, serine or metallo-proteinases. Further, since inhibitors are more useful than substrates in determining the nature of the catalytic groups, they have acquired special importance in the classification of endopeptidases. Barrett (1977) has reviewed the classification of tissue proteinases according to these guidelines. The rapidly increasing number of proteolytic enzymes now recognized to be present in cells and tissues was more recently reviewed by McDonald and Barrett (1980).

Some enzyme preparations first named as Cathepsins have subsequently been characterized as occuring in a number of forms. Kawamura (1975) found that pig kidney Cathepsin A is present in two different molecular sizes, designated Cathepsin A,L (large form) and Cathepsin A,S (small form) and isolated these two forms of Cathepsin A. Multiplicity of Cathepsin A was also reported by Matsuda and Misaka (1975). Cathepsin B, origionally defined as the

enzyme from bovine spleen deamidating benzoylarginine amide (Tallan, et al, 1952), was later shown to be two quite distinct enzymes of 25,000 and 52,000 daltons (Otto, 1971), which were named Cathepsin B1 and B2 respectively. Cathepsin B2 has since been renamed as Lysosomal Carboxypeptidase B and Cathepsin B1 is now known simply as Cathepsin B. Later work (Takahashi, et al, 1979) described three distinct forms of Cathepsin B, each of 29,000 daltons molecular weight, and suggested that two of the forms are produced by in vivo processing of the third. More recently, Mort (1983) characterized a latent cysteine proteinase from ascitic fluid as a high molecular weight form of Cathepsin B. This latent form was composed of a single chain of 40,000 daltons as determined by SDS-polyacrylamide gel electrophoresis, and after pepsin activation gave a single band of 33,000 daltons.

Using isoelectric focusing, Cathepsin D has been resolved into three major forms with slightly differing isoelectric points. The three forms were shown to be immunologically identical by Dingle (1971). Later, Huang (1979) purified six isozymes of Cathepsin D from porcine spleen. One isozyme had a molecular weight of 100,000 daltons and showed immunoidentity with the other five isozymes having molecular weights of 50,000 daltons. One isozyme of 50,000 daltons was found to be a single chain polypeptide while the other four contained two polypeptide

chains of approximately 30,000 and 15,000 daltons. Huang suggested that the high molecular weight form is the probable precursor of the single chain form of 50,000 daltons, which in turn produces the two chain isozymes. Puizdar (1981) further reported evidence for the existence of a Cathepsin D zymogen and suggested that it could be activated by a similar pH-dependent mechanism to that involved in the activation of pepsinogen by cleavage of a 5,000 dalton activation peptide.

In this thesis I describe the presence of a proteinase in bovine spleen that differs significantly from tissue proteinases previously described. Most notable properties are its high apparent molecular weight of approximately 395,000 daltons, a pH optimum of 3.2 when assayed against hemoglobin and the beta-chain of Insulin as substrates, and the dependence on thiol group activation for the proteolytic activity. Additional work is presented suggesting a lysosomal distribution for this enzyme. With this combination of properties being unique to this enzyme, it is proposed that herein is described a new high molecular weight thiol proteinase.

CHAPTER II

MATERIALS AND METHODS

Fractionation of Whole Organ Homogenates

The homogenization of the tissue, and the extraction and initial partial purification of the enzyme through acid precipitation and acetone fractionation steps, was by a modification of the methods of Barrett (1973). Bovine spleen, obtained fresh from a slaughterhouse and kept frozen until use, was blended in a Waring blender with two parts volume/weight of a solution containing 1% NaCl, 2% n-butanol, 0.2% Triton X-100 and 1 mM EDTA. The pH was adjusted to 6.1 using 5 M sodium formate pH 2.8, and the homogenate stirred slowly for one hour at 4°C. The homogenate was then centrifuged in a Sorvall centrifuge using a GSA head for 45 minutes at 7500 rpm (9150 x g). The supernatant was adjusted to pH 4.4, stirred for one hour and centrifuged as above. To the pH 4.4 supernatant was added 1.3 volumes of cold acetone, the precipitate collected by centrifugation, and the precipitate resuspended in 50 mM acetate buffer pH 5.3 containing 1 mM EDTA and 0.1 M NaCl. The resuspended acetone precipitate was stirred slowly for one hour at 4°C, then clarified by centrifugation as above. The supernatant solution was concentrated

using an Amicon PM-10 membrane and placed on a 2.6 x 92 cm column of Sepharose CL-6B equilibrated in 50 mM acetate pH 5.3 containing 1 mM EDTA and 0.1 M NaCl. Elution using the same buffer was at 24 ml/hr. Peak activity fractions were rechromatographed on a 1.6 x 92 cm column of Sepharose CL-4B and eluted at 14 ml/hr using the same buffer. Peak activity fractions from the Sepharose CL-4B column were pooled, concentrated on an Amicon PM-10 membrane and dialyzed against 50 mM acetate pH 5.3.

Purification and Recovery

Purification and recovery at each procedural step was determined using a modification of the proteinase assay of Anson (1939). To 50 ul of enzyme in 50 mM acetate buffer pH 5.3 was added either 50 ul of a solution containing 30 mM dithiothreitol (DTT) and 15 mM EDTA or 50 ul of 0.3 M $HgCl_2$. The mixture was incubated at room temperature for 15 minutes before addition of 50 ul of 1.0 M formate buffer pH 3.0 and 150 ul of 2% twice recrystallized bovine hemoglobin (Sigma) in water. The mixture was incubated at $37^{o}C$ for 30 minutes and the reaction stopped by addition of 1.5 ml of 3% trichloroacetic acid. After centrifugation, the absorbance of the supernate was read at 280 nm. Appropriate blanks were run to account for the A_{280} contribution of the enzyme and the substrate. Protein was determined by the method of Lowry (1951).

Purification fractions and column eluates were assayed for proteinase activity in the presence and absence of 5 mM dithiothreitol to determine the thiol dependency of the enzymatic activity (Kooistra, et al, 1982). In addition, assay of column eluate fractions for other known peptidases provided a basis for differentiating our enzyme from previously described ones. Thus, assays for Cathepsins B (Bajkowski and Frankfater, 1975), C (McDonald, et al, 1969) and D (Anson, 1939) were utilized.

Fractionation of Rat Livers

Six female rats were sacrificed by decapitation and the livers immediately excised and placed in ice cold saline. The livers were homogenized and fractionated in a manner identical to that used for bovine spleen through the preparation, resuspension and concentration of the acetone precipitate. The concentrated sample was chromatographed on a column of Sephadex G-200 using 50 mM acetate buffer pH 5.3 containing 1 mM EDTA and 0.1 M NaCl. Eluant fractions were assayed for Hemoglobin Hydrolase activity in the presence of either 5 mM dithiothreitol or 50 mM Hg^{2+} and for Cathepsin C activity (McDonald, et al, 1969).

Differential Centrifugation of Rat Liver Homogenates

The livers of freshly sacrificed rats were perfused with cold 0.25 M sucrose, homogenized in the same solution using three passes of a Kontes Teflon homogenizer, and

differentially centrifuged according to the method of De Duve (1955). The scheme used was a slight modification of the method of De Duve and is illustrated in Figure 1. Pellets obtained throughout the centrifugation scheme were assayed for the following subcellular marker enzymes: Cytochrome Oxidase (Cooperstein and Lazarow, 1951), Glucose-6-Phosphatase (De Duve, et al, 1955) (Fiske and Subbarow, 1925), and Acid Phosphatase (Berthet and De Duve, 1952). Protein in each fraction was determined using the Folin-Lowry (1951) method.

Isoelectric Focusing of Peak Activity Fraction

A sample of the enzyme was subjected to isoelectric focusing using a Brinkmann horizontal electrophoresis apparatus according to manufacturer's instructions. The sample was applied to a 20 x 20 cm glass plate coated with a mixture of Sephadex G-200 SF in water, 3 ml of pH 4-6 LKB ampholine, 0.04 g L-lysine and 0.04 g L-arginine. 200 V was applied for 16 hours followed by 500 V for 4 hours. Protein bands were blotted onto a strip of Whatman No. 2 filter paper and the adsorbed protein fixed in 10% trichloroacetic acid. The acid was washed from the paper using 60/133/20 Methanol/Water/Acetic Acid and the paper then stained with 0.2% Coumassie Blue in 50/50/10 Methanol/Water/Acetic Acid and destained with 33/66/10 Methanol/Water/Acetic Acid.

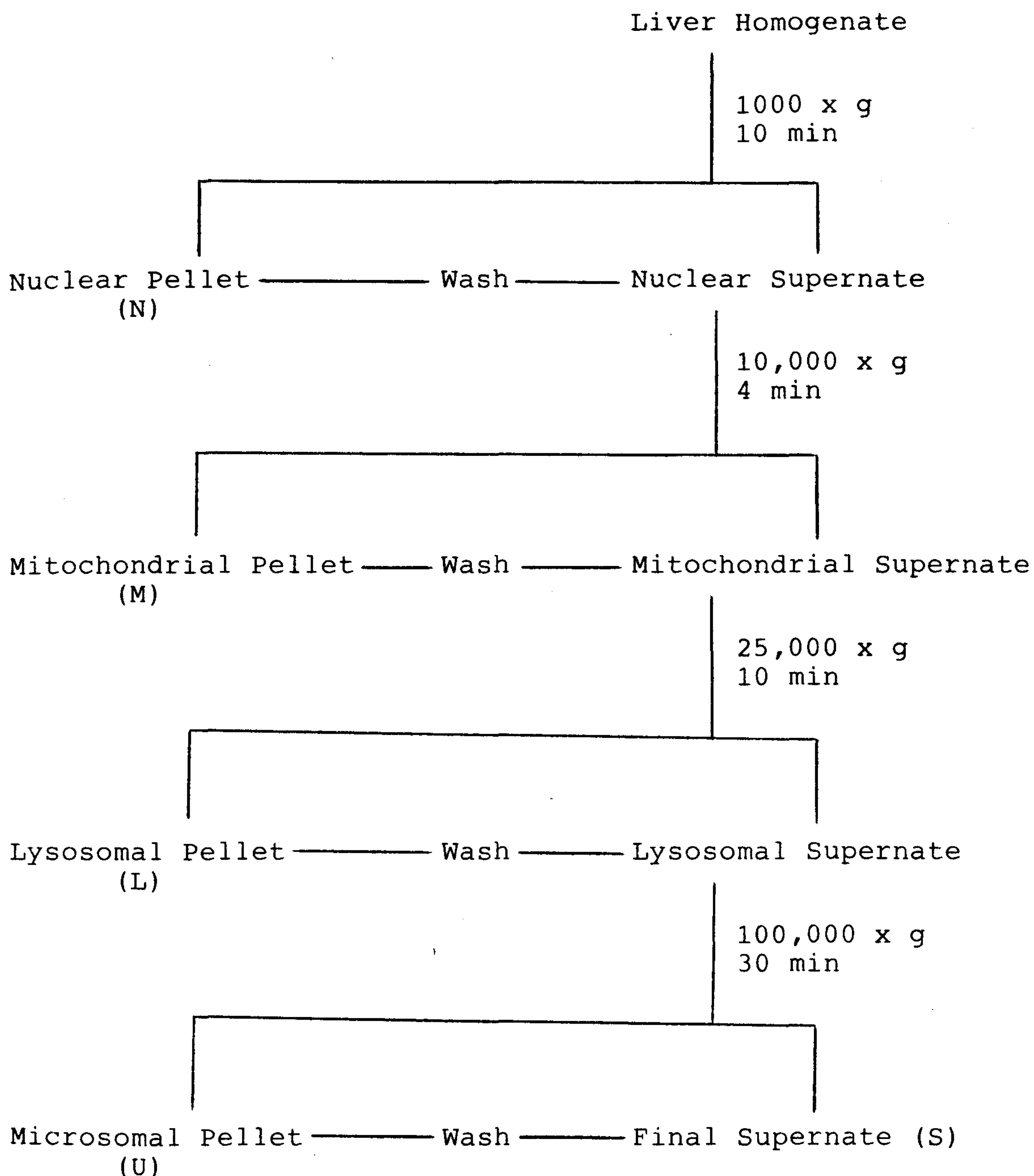


Figure 1. Differential Ultracentrifugation of Rat Liver Homogenate.

A Beckman Model J ultracentrifuge equipped with a T-50 head was used. Pellets were twice resuspended in 0.25 M sucrose, recentrifuged and the supernates combined.

Polyacrylamide Gel Electrophoresis

Polyacrylamide gel electrophoresis was performed according to the methods of Davis (1964). 30 mm stacking gels containing 2.5% acrylamide in 0.1 M Tris buffer pH 6.7 were prepared over 85 mm running gels containing 4% acrylamide in 0.4 M Tris buffer pH 8.9. Samples were layered on the gels and the gels subjected to 25-30 ma per tube for 70 minutes. The reservoir buffer contained 5 mM Tris and 0.04 M glycine adjusted to pH 8.3. Gels were stained with 1% Amido Black in 7% acetic acid for 1 hour and destained with 7% acetic acid.

Sodium Dodecyl Sulfate Polyacrylamide Gel Electrophoresis

Sodium dodecyl sulfate polyacrylamide gel electrophoresis was performed according to the method of Shapiro (1967) and molecular weights of the bands were determined as described by Weber and Osborn (1969). Samples were pretreated in 0.01 M phosphate buffer containing 5% SDS and 2% mercaptoethanol by placing them in a boiling water bath for 3 minutes. Some samples were alkylated with iodoacetate before electrophoresis as described by Weber (1972). Pharmacia molecular weight standards containing Thyroglobulin 330,000, Half Ferritin 220,000, Albumin 67,000, Catalase 60,000, and Lactate Dehydrogenase 36,000 daltons were prepared for electrophoresis in an identical fashion. 4% acrylamide gels were subjected

to 25-30 ma per gel for 70 minutes. The reservoir buffer contained 5 mM Tris and 0.04 M glycine adjusted to pH 8.3. Gels were stained for 10 minutes in 50/50/10 MeOH/H_2O/HOAc containing 0.25% Coumassie Blue and destained for 24 hours using a Bio-Rad diffusion destainer containing 2/35/3 MeOH/H_2O/HOAc.

Determination of Carbohydrate Content

Carbohydrate content of enzyme preparations containing between 1-2 mg protein per ml were estimated using the Anthrone method as described by Dische (1962). The method was standardized using D-(+)-glucose from 0-100 ug/ml.

Molecular Weight Determination of the Enzyme

Molecular weight of the enzyme was determined by gel filtration through a 1.6 x 87 cm calibrated column of Sepharose CL-4B equilibrated in 50 mM acetate buffer pH 5.3 containing 1 mM EDTA and 0.1 M NaCl. Elution at 14 ml/hr was monitored at 280 nm. Molecular weight was determined by comparing elution volume with the elution volumes of the following standards: Thyroglobulin 670,000, B-Galactosidase 520,000, Xanthine Oxidase 275,000, and Catalase 250,000 daltons.

Assay for Proteolytic Activity Using the Beta-Chain of Insulin and Separation of Peptides by Cation Exchange Chromatography

Enzyme in 50 mM acetate buffer pH 5.3 was preincubated with 15 mM dithiothreitol and 7.5 mM EDTA for 15 minutes at room temperature. To 1 ml of enzyme-activator mixture was added 1.4 ml beta-Insulin in 0.5 M formate buffer pH 3.0. The final mixture contained approximately 0.1 ug of protein as enzyme, and was 2.9 mM in beta-Insulin, 6.2 mM in dithiothreitol and 3 mM in EDTA. The assay mixture was incubated at 37°C for 3 hours and the reaction stopped by chilling in an ice bath.

Peptides in the reaction mixture were separated by cation exchange chromatography according to the method of Schroeder (1967). The pyridine-acetate gradient was from 0.2 M pyridine pH 3.1 to 2.0 M pyridine pH 5.0, and was used to elute the peptides from a Bio-Rad AG50W-X2 cation exchange column. The apparatus is diagramed in Figure 2. Collected fractions were assayed by the ninhydrin method of Moore and Stein (1954) using the automated adaptation of Lenard (1965). Following initial ninhydrin determination, alkaline hydrolysis was performed on some eluates as described by Hirs (1956).

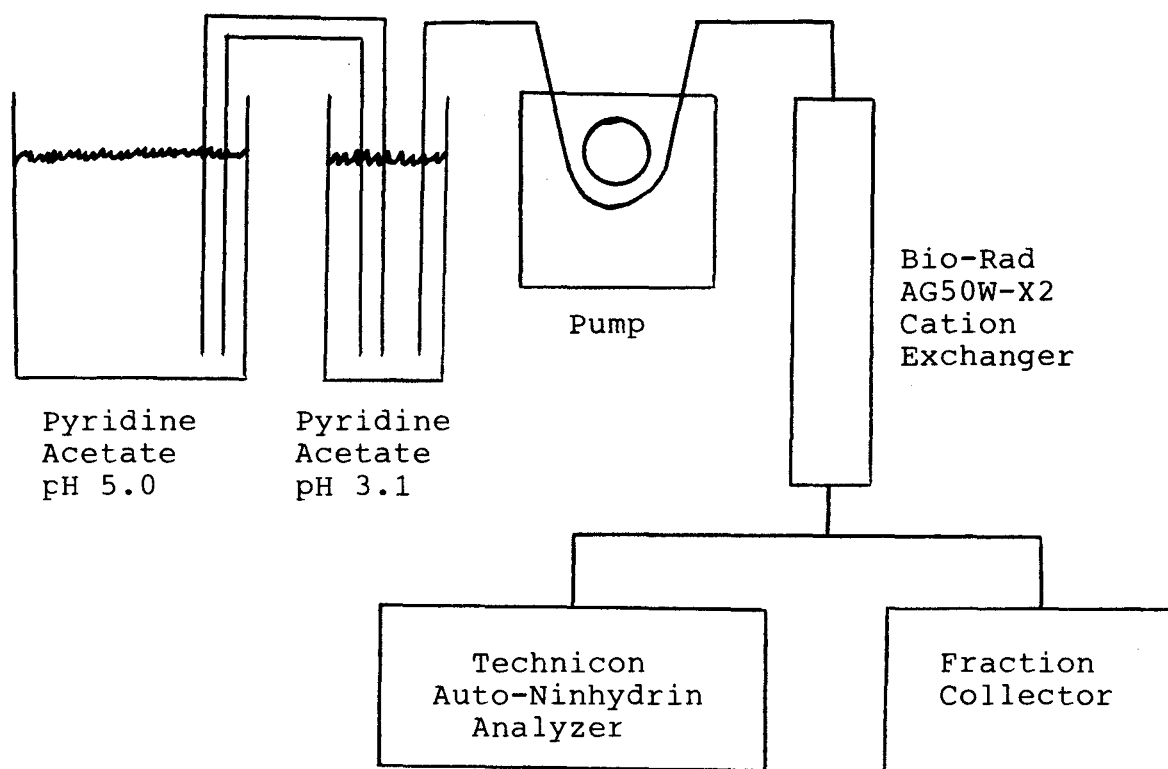


Figure 2. Apparatus for Cation Exchange Chromatography with Automated Ninhydrin Determination of Eluates.

The diameter of the pH 5.0 reservoir was twice that of the pH 3.1 reservoir, thereby developing a concave gradient. The ninhydrin analyzer required approximately one tenth of the column eluate. Total volume of the gradient was 330 ml and was eluted at 9.5 ml/hr.

Assay for Proteolytic Activity Using the Beta-Chain of Insulin and Separation of Peptides by High Performance Liquid Chromatography

Approximately 2 ug of enzyme protein was activated in 15 mM dithiothreitol and 7.5 mM EDTA at room temperature before addition of the substrate. Beta-Insulin in 0.5 M formate pH 3.0 was added to a final concentration of 0.2 mM. The final assay contained 7.5 mM dithiothreitol and 3.75 mM EDTA in a total volume of 2.5 ml. Incubation of the final assay containing 0.5 ug of enzyme protein was at $37^{o}C$. Small aliquotes were removed from the assay mixture at timed intervals and quick frozen in dry ice-acetone to stop the reaction. Similar assays were also carried out in the absence of dithiothreitol and EDTA.

Assay aliquotes collected after various times of incubation were separated by high performance liquid chromatography using a C_{18} reversed phase column obtained from Waters Associates. Elution was by a 60 minute linear gradient of 0-70% methanol in 0.1% phosphoric acid. The flow rate of the column was 1 ml/min and elution was monitored at 214 nm.

Inhibitors of Proteolytic Activity

The effect of proteolytic inhibitors on the degradation of beta-Insulin by the enzyme was studied. 2 ug of enzyme protein was incubated with various concentrations of

the inhibitor at room temperature for 15 minutes. Dithiothreitol and EDTA were added to concentrations of 15 mM and 7.5 mM respectively and the mixture incubated at room temperature for an additional 15 minutes. Beta-Insulin in 0.5 M formate buffer pH 3.0 was added to a final concentration of 0.2 mM and the assay carried out at 37°C. Aliquotes were taken out at timed intervals and quick frozen in dry ice-acetone. Final assay concentrations were as previously described in high performance liquid chromatography experiments.

<u>Assay with Glycylglycyl-L-phenylalanyl-L-phenylalanine Ethyl Ester as Substrate</u>

Enzyme was preincubated with dithiothreitol and EDTA as previously described. Glycylglycyl-L-phenylalanyl-L-phenylalanine ethyl ester (Sigma) in 0.5 M formate pH 3.0 was added to a final concentration of 2.4 mM. The final assay contained 5 mM dithiothreitol, 2.6 mM EDTA and 0.8 ug enzyme protein in a total volume of 2.7 ml. The reaction was carried out at 37°C with aliquotes removed and quick frozen in dry ice-acetone at timed intervals. Separation of peptides was by high performance liquid chromatography using a 0-70% methanol gradient in 0.1% phosphoric acid as previously described. Appropriate assay blanks were also run under similar conditions.

Effect of Adenosine Triphosphate (ATP) on the Degradation of Beta-Insulin by the Enzyme

The effect of preincubating the enzyme in the following activating solutions was studied: (1) 15 mM dithiothreitol (DTT); (2) 15 mM DTT and 10 mM ATP; (3) 15 mM DTT, 10 mM ATP and 10 mM Mg^{2+}; (4) 10 mM ATP and 10 mM Mg^{2+}; (5) 10 mM Mg^{2+}. 150 ul of enzyme in 50 mM acetate pH 5.3 was incubated with 150 ul of activator at the concentrations listed. 300 ul of beta-Insulin in 0.5 M acetate pH 6.3 was added to start the assay. Thus, in those assays where included, final concentrations of the various activators were 7.5 mM dithiothreitol, 5 mM ATP, and 5 mM Mg^{2+}. The assay contained approximately 0.8 ug of enzyme protein with beta-Insulin at a final concentration of 0.2 mM. Incubation was at 37°C for 30 minutes and reaction was stopped by freezing in dry ice-acetone. Resulting peptides were separated by high performance liquid chromatography in a 40-60% methanol gradient in 0.1% phosphoric acid run over a period of 30 minutes at a column flow of 1 ml/min. A C_{18} reversed phase column was used.

CHAPTER III

EXPERIMENTAL RESULTS

Purification and Recovery

Typical data for recovery and purification of Hemoglobin Hydrolase activity from bovine spleen through the various purification steps are given in Table 1. The crudeness of the pH 6.1 supernate and the large amount of protein present, made it difficult to do a correction for the blank contribution to the hemoglobin assay of this purification step. For this reason, purification and yield data are calculated based on the pH 4.4 supernate. Final yield of mercury sensitive Hemoglobin Hydrolase activity ranged from 2-4%, with purification ranging from 20-40 fold in various preparations of enzyme from bovine spleen.

Elution From Sepharose CL-6B and Sepharose CL-4B Columns

The elution pattern of various components from a Sepharose CL-6B column is shown in Figure 3. A high molecular weight Hemoglobin Hydrolase activity is clearly separated out in the early eluting fractions from a large peak of Hemoglobin Hydrolase activity eluting in the smaller molecular weight ranges. The higher molecular weight Hemoglobin Hydrolase activity is completely inhi-

Table 1. PURIFICATION OF HIGH MOLECULAR WEIGHT PROTEINASE FROM BOVINE SPLEEN

Purification Fraction	Total Protein (mg)	Total[a] Activity (DTT)	Total[b] Activity (Mercury Sensitive)	Specific[c] Activity (DTT)	Specific[c] Activity (Mercury Sensitive)	% Yield Mercury Sensitive Activity
pH 6.1 Supernate	16,044	794	---	0.03	---	---
pH 4.4 Supernate	6,970	2777	1476	0.26	0.18	100
Solubilized Acetone Precipitate	533	896	691	1.12	0.52	43
Peak Tube Sepharose CL-6B	6.3	37	36	3.87	3.55	4
Pooled Tubes Sepharose CL-4B	6.3	24	24	2.50	2.51	3

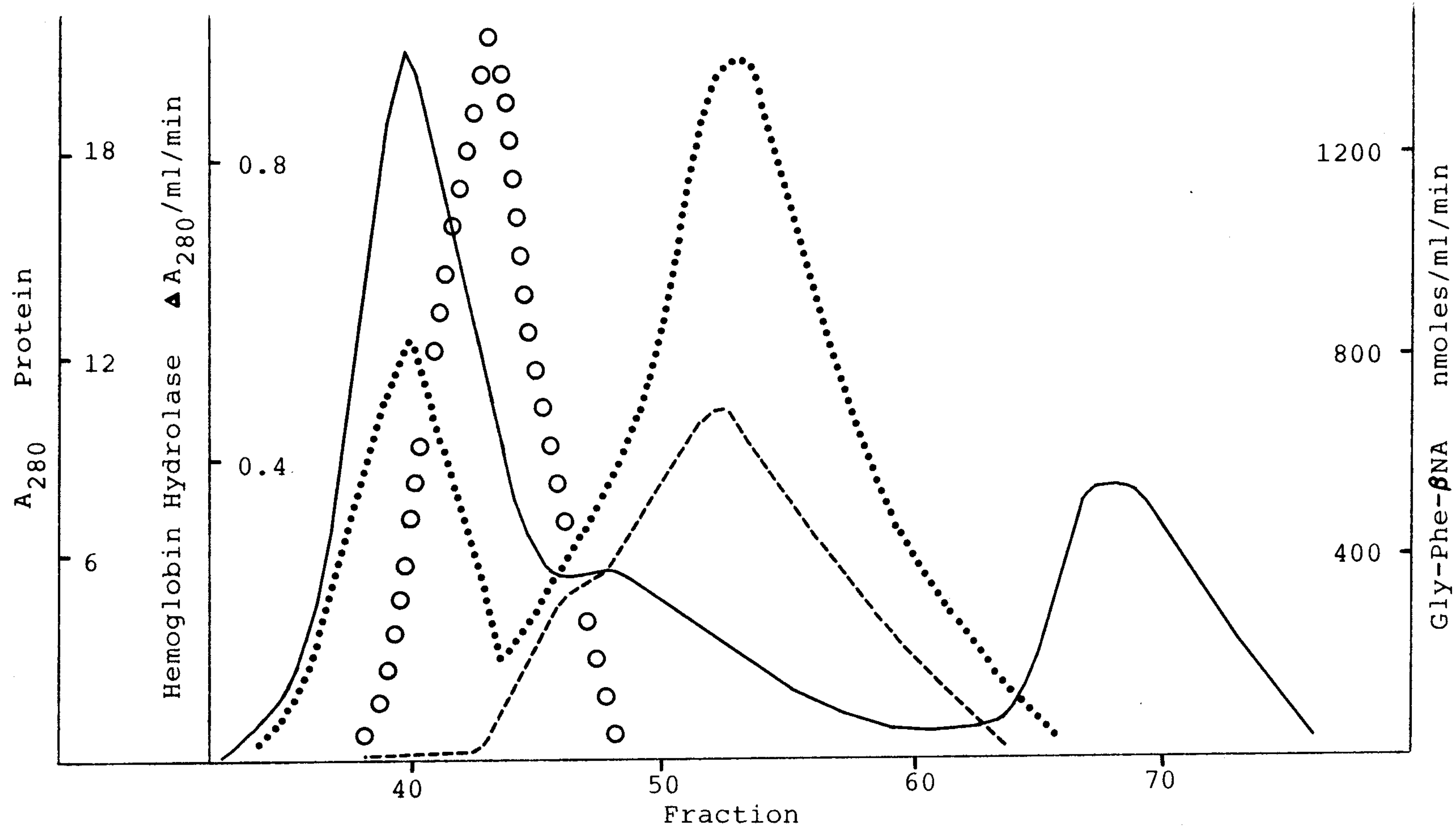
[a] DTT, dithiothreitol. Activity in Hemoglobin Hydrolase assay expressed as ΔA_{280}/ml/min.

[b] Mercury sensitive activity obtained by subtracting activity with mercury present from activity obtained with DTT.

[c] Specific activity expressed as ΔA_{280}/ml/min/mg enzyme protein.

Figure 3. Chromatography of Resolubilized Acetone Precipitate From Bovine Spleen on Sepharose CL-6B.

(———) Protein at 280 nm. (•••••) Hemoglobin Hydrolase with dithiothreitol. (------) Hemoglobin Hydrolase with mercury. (O O O) Glycyl-phenylalanine-β-naphthylamide, for Cathepsin C. Conditions of chromatography and enzyme assays are described in the text.



A280 Protein
18
12
6
Hemoglobin Hydrolase ΔA280/ml/min
0.8
0.4
1200
800
400
Gly-Phe-βNA nmoles/ml/min
40
50
60
70
Fraction

bited upon substitution of 0.3 mM Hg^{2+} in the assay for the 10 mM dithiothreitol and 5 mM EDTA. The lower molecular weight Hemoglobin Hydrolase activity peak however, is only inhibited approximately 50% by the same substitution. Assay of column eluates for Cathepsin C activity places the high molecular weight Hemoglobin Hydrolase activity at an apparent molecular weight in excess of 210,000 which is the reported molecular weight of Cathepsin C.

Peak activity tubes of the high molecular weight Hemoglobin Hydrolase activity were pooled, concentrated on an Amicon PM-10 membrane and rechromatographed on a column of Sepharose CL-4B. The A_{280} elution pattern is shown in Figure 4. Assay of the eluant peaks indicated that only the high molecular weight peak had Hemoglobin Hydrolase activity and this activity was completely inhibited by mercury.

<u>High Molecular Weight Proteinase in Rat Liver</u>

Rats were sacrificed by decapitation and livers excised and prepared in a manner identical to that for bovine spleen as described in methods. Hemoglobin assay of the pH 4.4 supernatant indicated that over 60% of the Hemoglobin Hydrolase activity present was sensitive to mercury. An acetone precipitate was prepared, resuspended and concentrated as described for bovine spleen. The

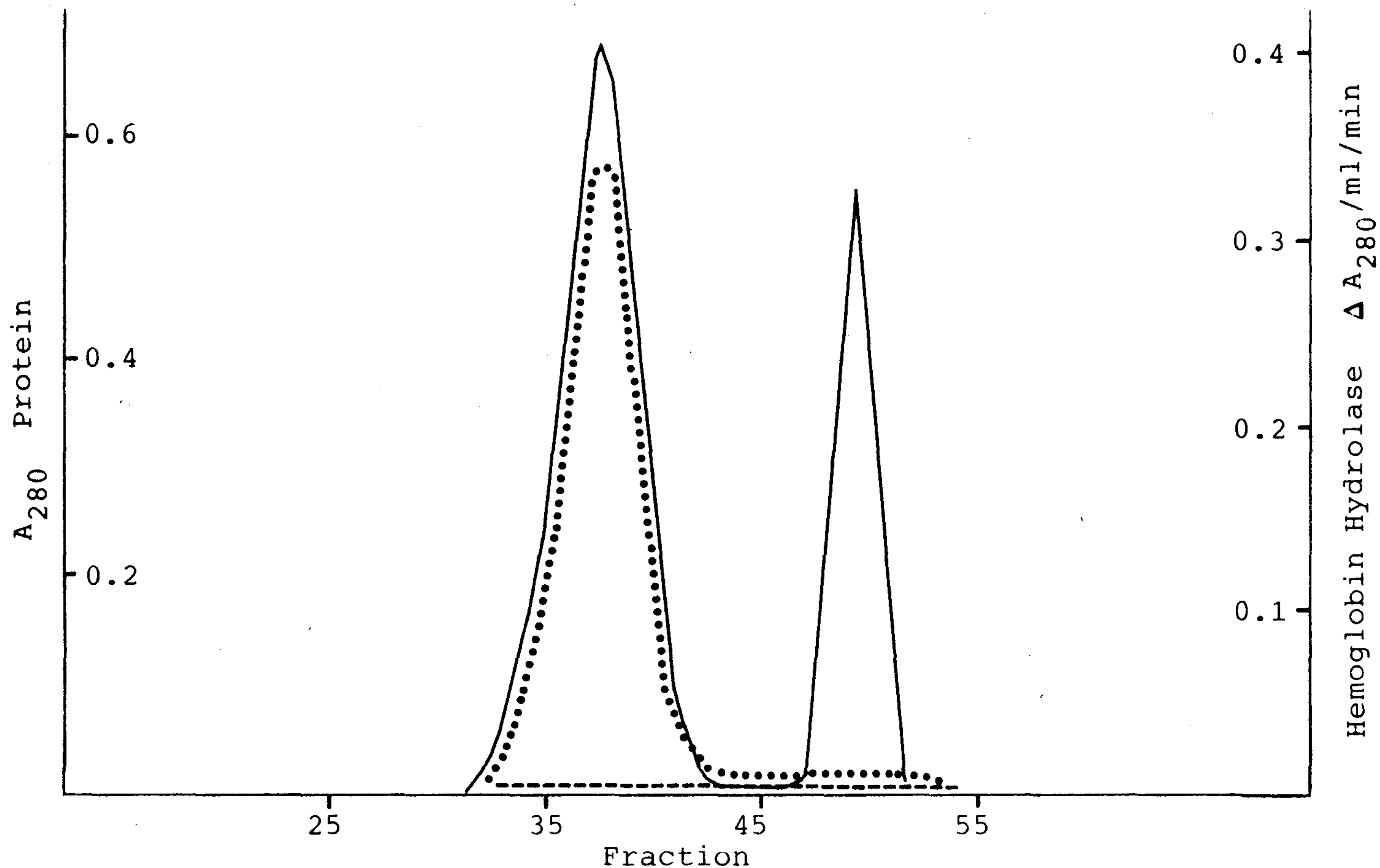


Figure 4. Chromatography of Sepharose CL-6B Activity Peak Obtained From Bovine Spleen on Sepharose CL-4B.

(———) Protein at 280 nm. (●●●●●) Hemoglobin Hydrolase with dithiothreitol. (------) Hemoglobin Hydrolase with mercury. Conditions of chromatography and enzyme assays are described in the text.

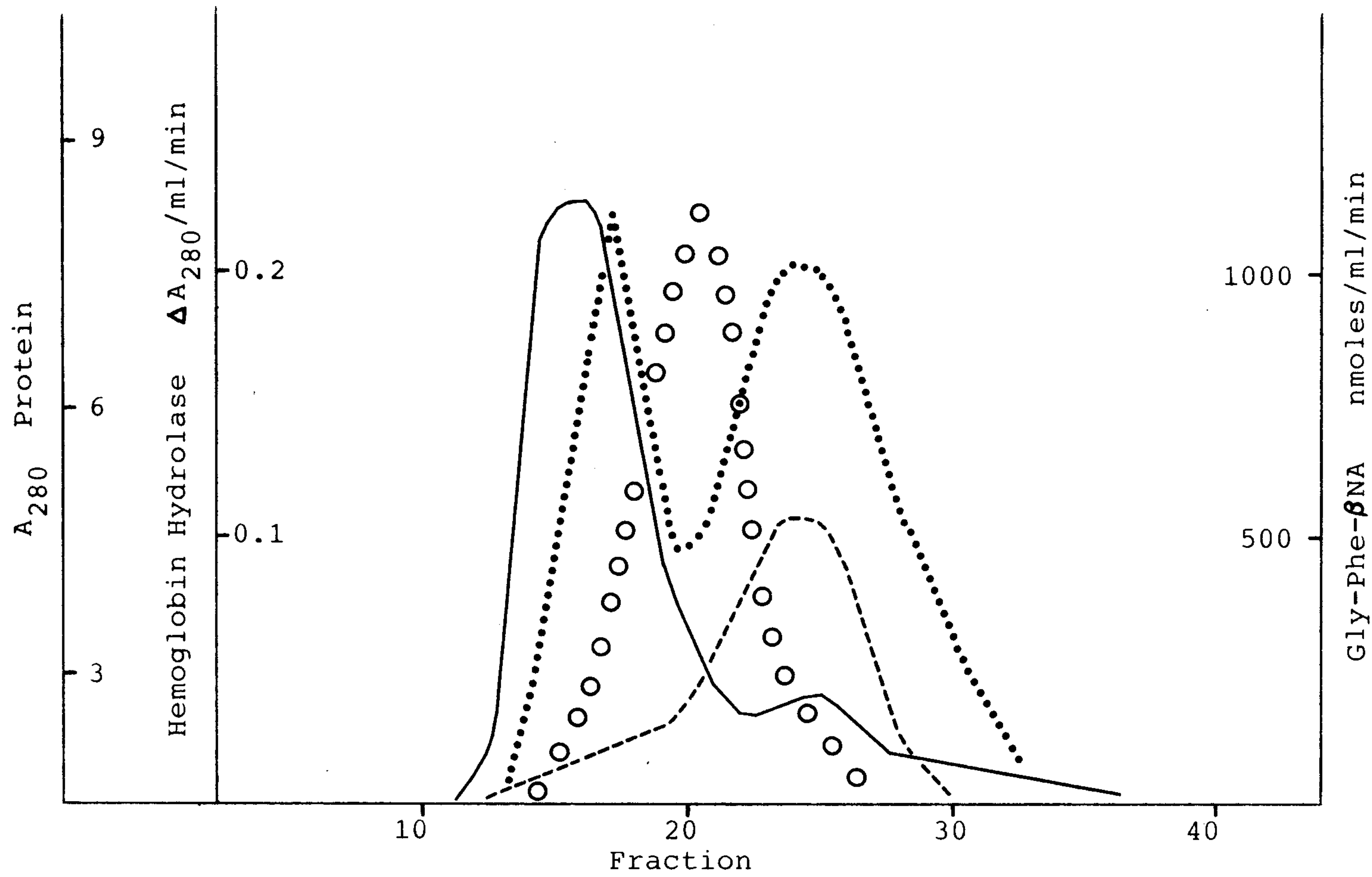
sample was then chromatographed on a 1.6 x 30 cm column of Sephadex G-200 using 50 mM acetate buffer pH 5.3 containing 1 mM EDTA and 0.1 M NaCl at a flow rate of 12 ml/hr. Elution profiles for Hemoglobin Hydrolase activity and for Cathepsin C activity are shown in Figure 5. Here again is seen a high molecular weight Hemoglobin Hydrolase activity eluting at a position prior to that of Cathepsin C, indicating an apparent molecular weight in excess of 210,000 daltons.

Differential Ultracentrifugation of Perfused Rat Liver

Recovery of various subcellular component marker enzymes during differential ultracentrifugation of perfused rat liver homogenate is shown in Figure 6. This method of illustration allows the comparison of patterns of enzyme localization in the various fractions obtained after ultracentrifugation. The sulfhydryl dependent Hemoglobin Hydrolase activity displayed a pattern of localization similar to that of Acid Phosphatase, thus suggesting a light mitochondrial, or lysosomal localization for the enzyme. Bars in the diagram indicate the range of relative specific activity obtained in a number of experiments. The percent of total recovered activity of the marker enzymes for each subcellular fraction in one typical experiment is shown in Table 2.

Figure 5. Chromatography of Resolubilized Acetone Precipitate From Rat Liver on Sephadex G-200.

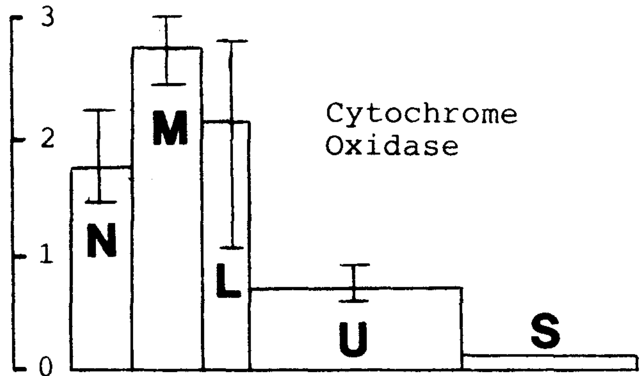
(———) Protein at 280 nm. (••••••) Hemoglobin Hydrolase with dithiothreitol. (------) Hemoglobin Hydrolase with mercury. (OOO) Glycyl-phenylalanine-β-naphthylamide, for Cathepsin C. Conditions of chromatography and enzyme assays are described in the text.

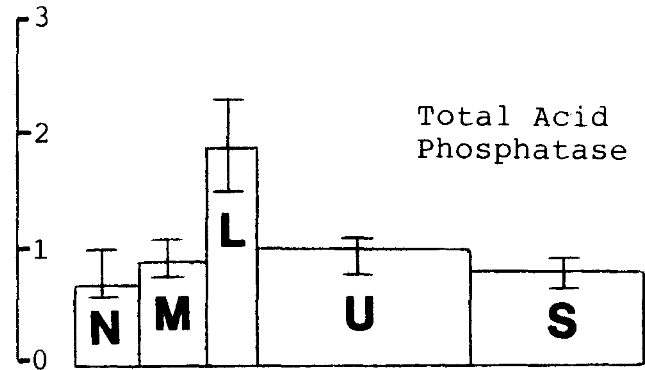


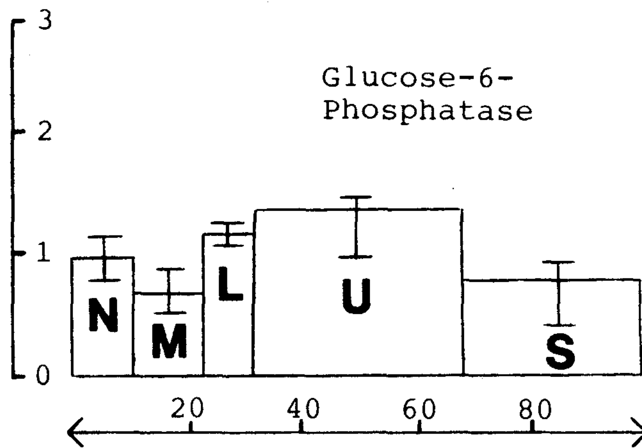
A280 Protein
9
6
3
Hemoglobin Hydrolase ΔA280/ml/min
0.2
0.1
1000
500
Gly-Phe-βNA nmoles/ml/min
10
20
30
40
Fraction

Figure 6. Recovery of Subcellular Component Marker Enzymes During Differential Ultracentrifugation of Perfused Rat Liver Homogenate.

The width of each bar along the horizontal axis indicates the percent of the total protein recovered in each subcellular fraction: (N) Nuclear Debris; (M) Heavy Mitochondrial; (L) Light Mitochondrial; (U) Microsomal; (S) Final Supernate. Total width of the horizontal axis corresponds to 100% of the recovered protein. The vertical axis describes the ratio of percent total activity recovered in each fraction to the percent total protein recovered in each fraction. Error bars indicate the range of values obtained in replecate preparations.


Cytochrome
Oxidase
3
2
1
0
N
M
L
U
S


Total Acid
Phosphatase
3
2
1
0
N
M
L
U
S


Glucose-6-
Phosphatase
3
2
1
0
N
M
L
U
S
20
40
60
80

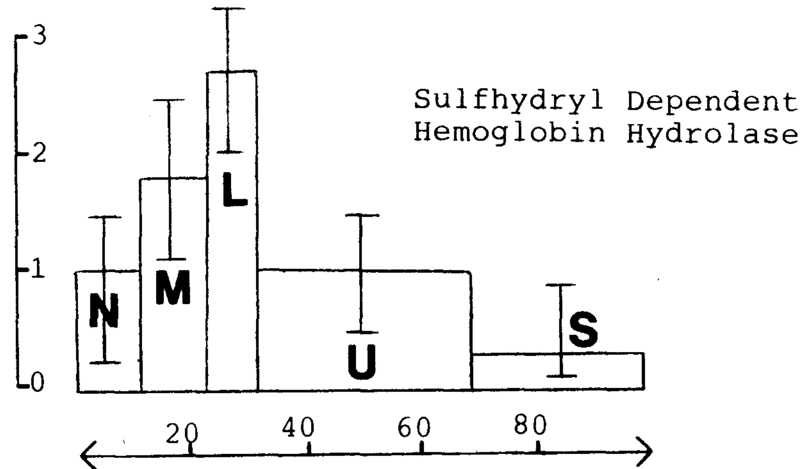

Sulfhydryl Dependent
Hemoglobin Hydrolase
3
2
1
0
N
M
L
U
S
20
40
60
80

Table 2. SUMMARY OF RECOVERY OF MARKER ENZYME ACTIVITY IN SUBCELLULAR FRACTIONS FROM RAT LIVER

Fraction[a]	Percent Total Protein	Percent Cytochrome Oxidase	Percent Glucose-6-Phosphatase	Percent Total Acid Phosphatase	Percent Sulfhydryl Dependent Hemoglobin Hydrolase
(N)	11.2	19.5	9.4	7.6	0.0
(M)	11.5	34.0	8.6	10.3	17.6
(L)	8.9	17.7	10.9	16.8	29.5
(U)	37.5	27.0	45.9	38.9	53.3
(S)	31.2	1.4	24.8	25.6	0.0

[a]Fractions: (N) Nuclear Debris; (M) Heavy Mitochondrial; (L) Light Mitochondrial; (U) Microsomal; (S) Final Supernate.

Sephadex G-200 Chromatography of Lysosomal Proteins

Light mitochondrial pellets obtained by differential ultracentrifugation of six rat liver homogenates were lysed in a minimal volume of 1 mM acetate buffer pH 5.1 containing 0.2% Triton X-100 and maintained for 20 minutes at 4°C. The pooled lysates were centrifuged at 100,000 x g for 60 minutes. The supernate was dialyzed against 1 mM acetate buffer pH 5.1 and concentrated using an Amicon PM-10 membrane. 12.5 ml of concentrate was placed on a 2.5 x 89 cm column of Sephadex G-200 and eluted at 10 ml/hr with acetate buffer pH 5.3 containing 1 mM EDTA and 0.1 M NaCl. The elution of Hemoglobin Hydrolase activity assayed in the presence of either 5 mM dithiothreitol or 50 mM mercury is shown in Figure 7. As is seen with whole organ bovine spleen and rat liver preparations, a high molecular weight Hemoglobin Hydrolase activity elutes under the major protein peak, and this activity is sensitive to mercury.

pH Dependency Using Hemoglobin as Substrate

The pH dependency of the Sepharose CL-4B purified Hemoglobin Hydrolase activity from bovine spleen was determined in 1.0 M formate buffers over a range of pH using the hemoglobin assay essentially as previously described. Incubation was at 37°C for 30 minutes in the presence of 10 mM dithiothreitol and 5 mM EDTA. Trichloroacetic acid was added to a final concentration of 3% and, after cen-

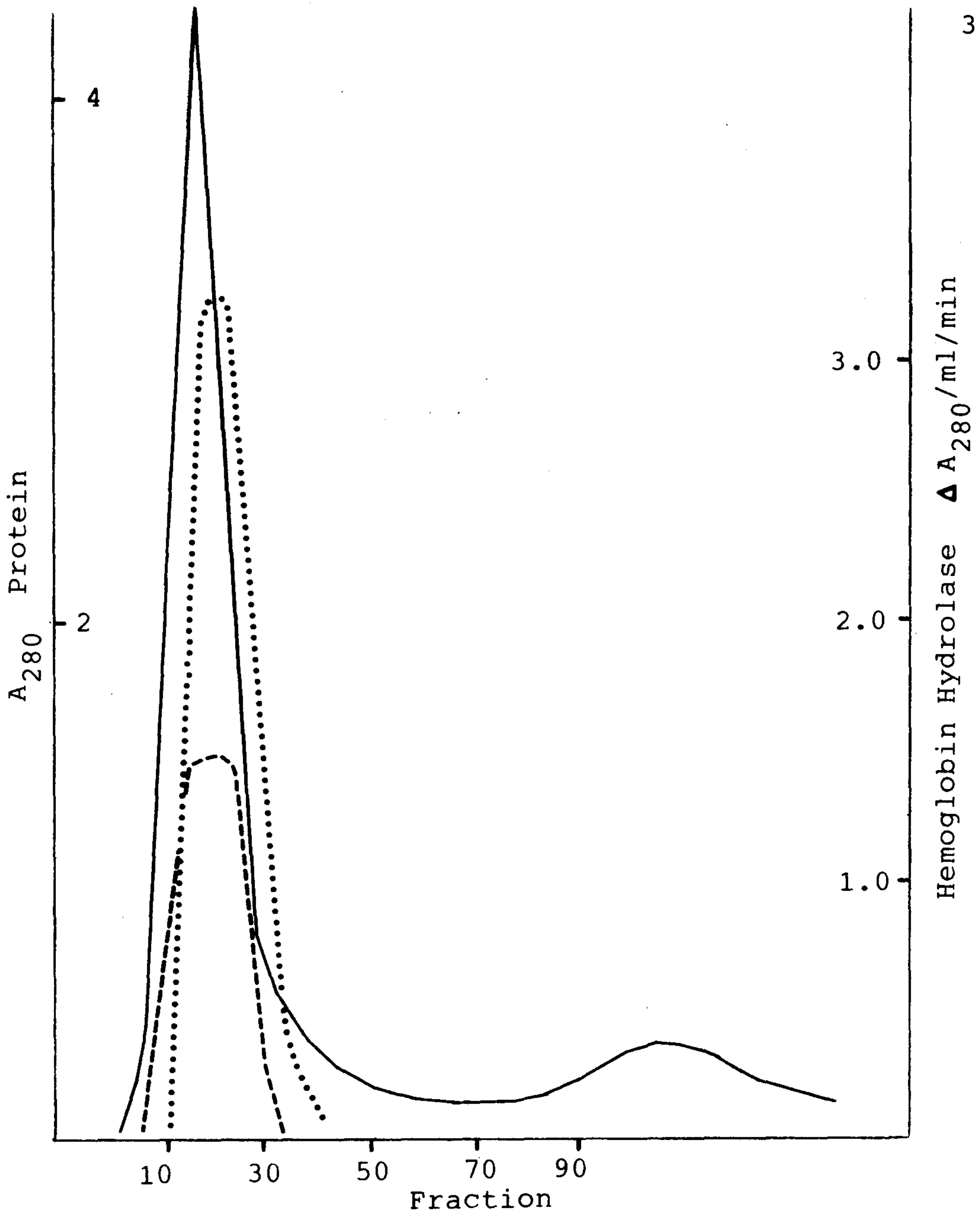


Figure 7. Sephadex G-200 Chromatography of Hemoglobin Hydrolase Activity From Lysosome Enriched Subcellular Fraction of Rat Liver.

(———) Protein at 280 nm. (•••••••) Hemoglobin Hydrolase with dithiothreitol. (-----) Hemoglobin Hydrolase with mercury. Conditions of chromatography and enzyme assays are described in the text.

trifugation , the absorbance of the supernatant was determined at 280 nm. Results are shown in Figure 8, indicating a pH optimum of approximately 3.4.

Polyacrylamide Gel Electrophoresis

The peak activity fractions from Sepharose CL-6B and Sepharose CL-4B columns of a bovine spleen preparation yielded single bands on polyacrylamide gel electrophoresis. The gels are shown in Figure 9.

Thin Layer Isoelectric Focusing

Thin layer isoelectric focusing of the peak activity fraction from a Sepharose CL-4B column of a bovine spleen preparation yielded a single band occurring at pH 4.95. The strip of filter paper used to visualize the thin layer plate is shown in Figure 10.

Molecular Weight Determination by Calibrated Sepharose CL-4B Column

A column of Sepharose CL-4B (1.6 x 87 cm) was equilibrated in 50 mM acetate pH 5.3 containing 0.1 M NaCl and 1 mM EDTA. Elution was at 14 ml/hr. A sample of Sepharose CL-4B purified high molecular weight sulfhydryl dependent proteinase eluted from this column at an elution volume equivalent to a molecular weight of 395,000 daltons. Results are plotted in Figure 11.

Figure 9. Polyacrylamide Gel Electrophoresis Under Non-Reducing Conditions of Peak Activity Fractions From Sepharose CL-6B and CL-4B Columns.

The enzyme source was bovine spleen. The method used was essentially that of Davis (1964) with conditions as described in the text.

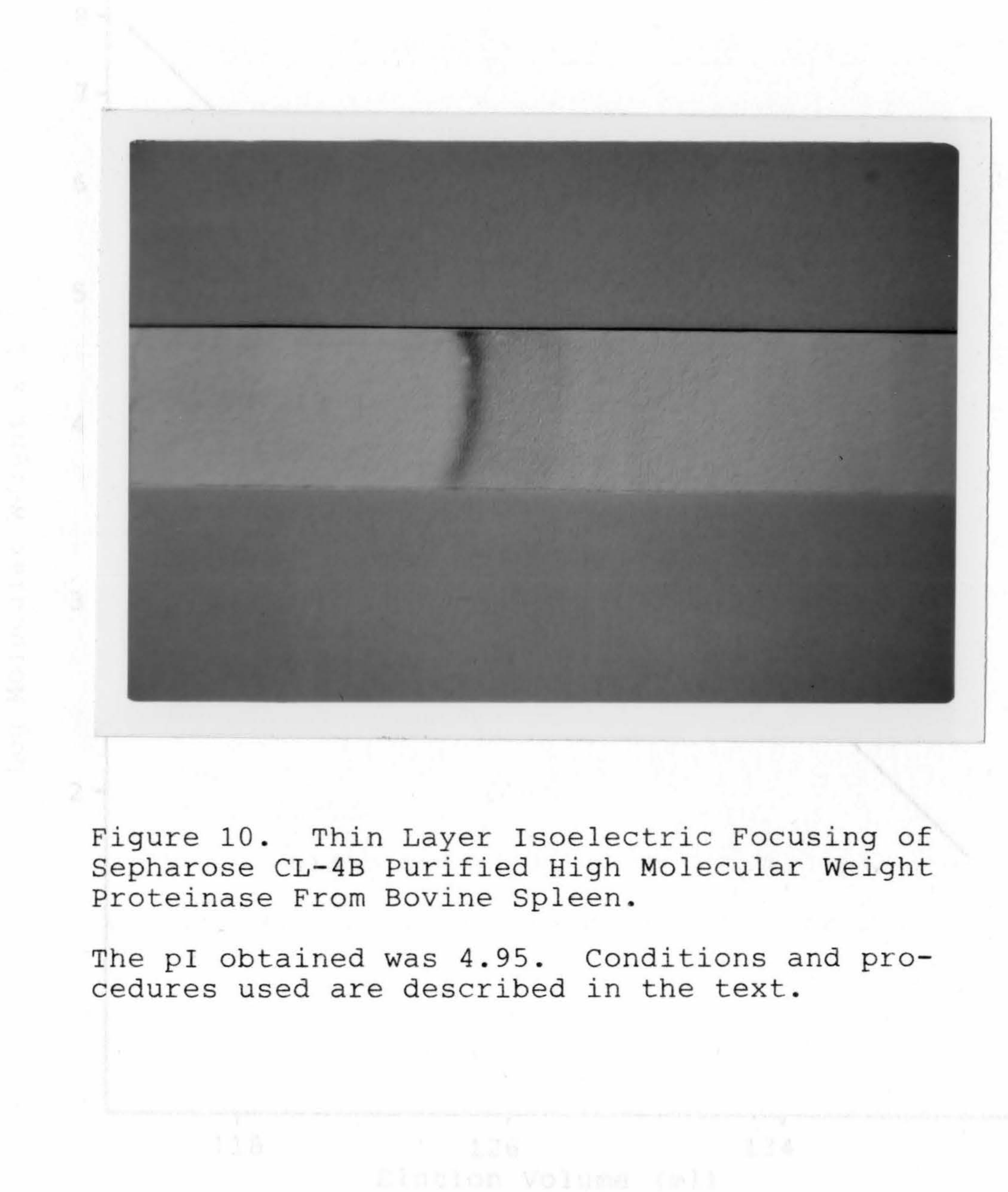


Figure 10. Thin Layer Isoelectric Focusing of Sepharose CL-4B Purified High Molecular Weight Proteinase From Bovine Spleen.

The pI obtained was 4.95. Conditions and procedures used are described in the text.

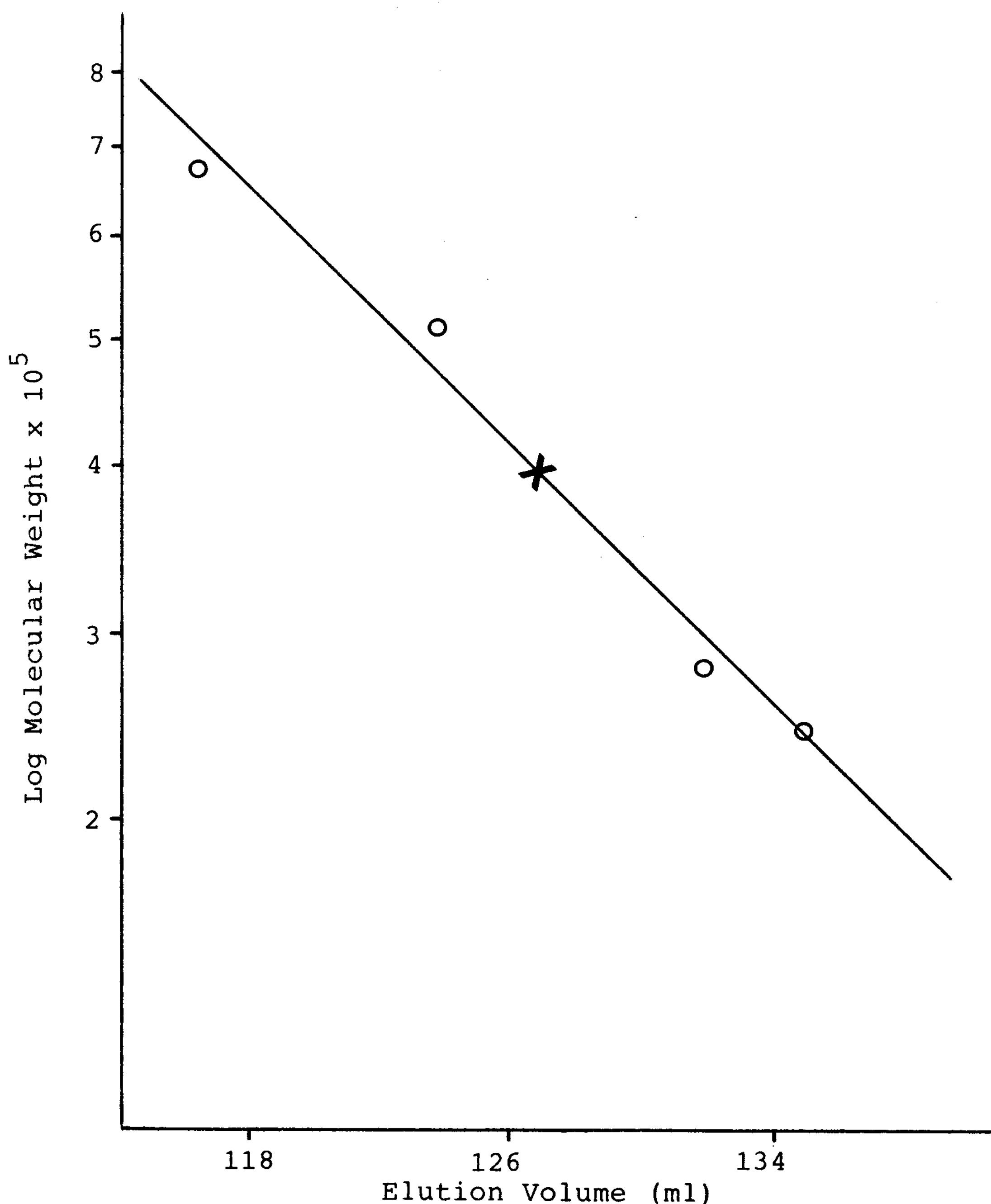


Figure 11. Molecular Weight Determination of High Molecular Weight Proteinase From Bovine Spleen By Chromatography on a Calibrated Sepharose CL-4B Column.

Molecular weight markers (O): Thyroglobulin, 670,000; B-Galactosidase, 520,000; Xanthine Oxidase, 275,000; Catalase, 250,000 daltons. Apparent molecular weight of the proteinase (X) is 395,000 daltons. Conditions of chromatography are described in the text.

Sodium Dodecyl Sulfate Polyacrylamide Gel Electrophoresis

Gels were run as described in methods using a Pharmacia molecular weight standard mixture containing Thyroglobulin 330,000, Half Ferritin 220,000, Albumin 67,000, Catalase 60,000, and Lactate Dehydrogenase 36,000 daltons. Simultaneously run samples of Sepharose CL-4B purified high molecular weight sulfhydryl dependent proteinase from bovine spleen gave three distinct bands corresponding to subunit molecular weights of 95,000, 60,000 and 40,000 daltons. A graph comparing log molecular weight to percent mobility of each band in the gels is shown in Figure 12. The stained gels are shown in Figure 13.

Carbohydrate Content

The Anthrone method of Dische (1962) was used to estimate the carbohydrate content in the peak activity fraction from a Sepharose CL-4B column of a bovine spleen preparation. Protein content of the enzyme solution was determined using the method of Lowry (1951), while the molecular weight of the enzyme was approximated to be 395,000 daltons. Carbohydrate content was determined to be 3% by weight based on a glucose standard. This weight of carbohydrate would be equivalent to approximately 55 residues of carbohydrate, as glucose, per molecule of enzyme.

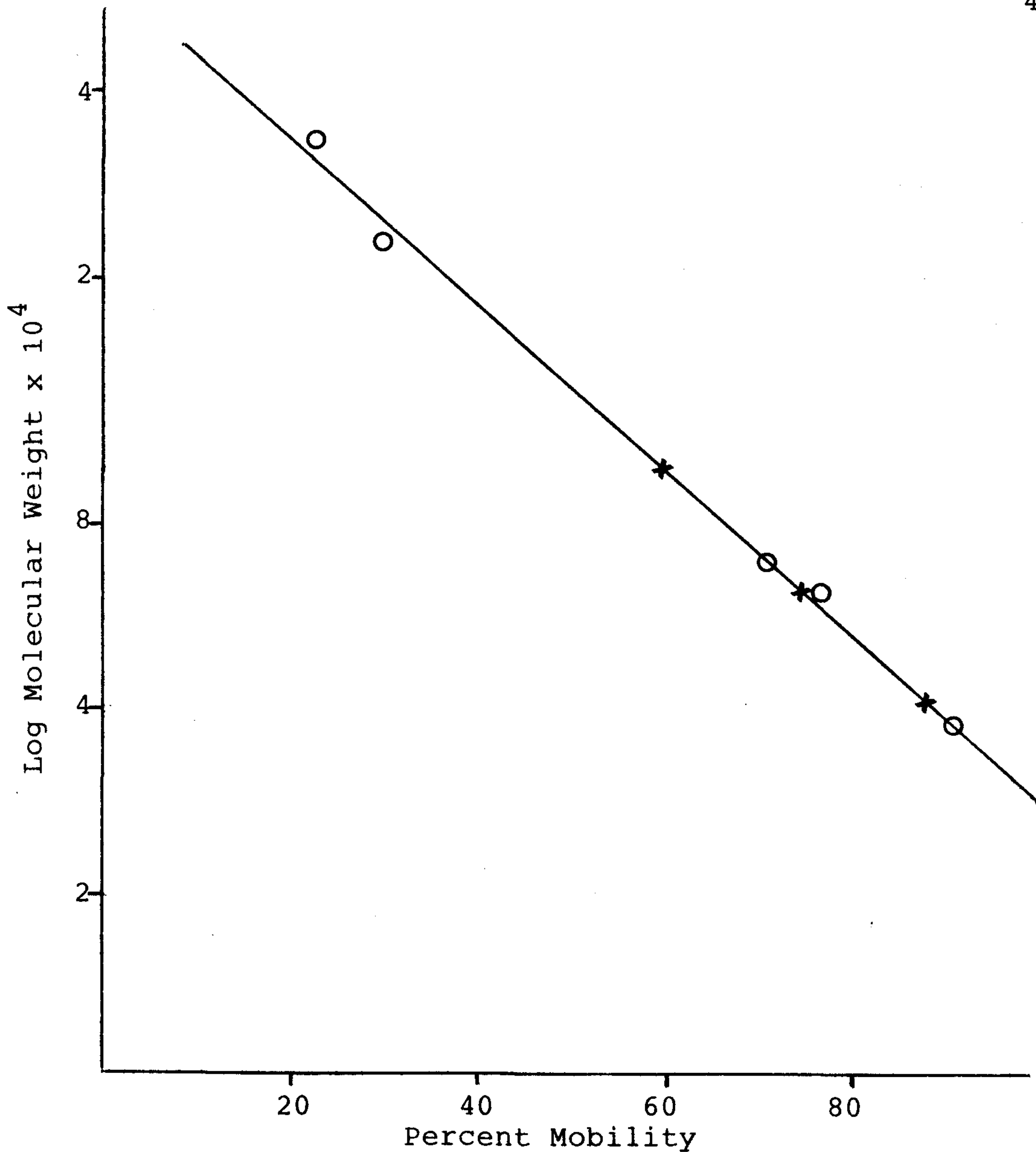


Figure 12. Sodium Dodecyl Sulfate Polyacrylamide Gel Electrophoresis of Marker Proteins and Treated High Molecular Weight Proteinase From Bovine Spleen.

Molecular weight markers: Thyroglobulin, 330,000; Half Ferritin, 220,000; Albumin, 67,000; Catalase, 60,000; Lactate Dehydrogenase, 36,000. Apparent molecular weight of bands from the high molecular weight proteinase are 95,000, 60,000 and 40,000 daltons. Conditions of electrophoresis are described in the text.

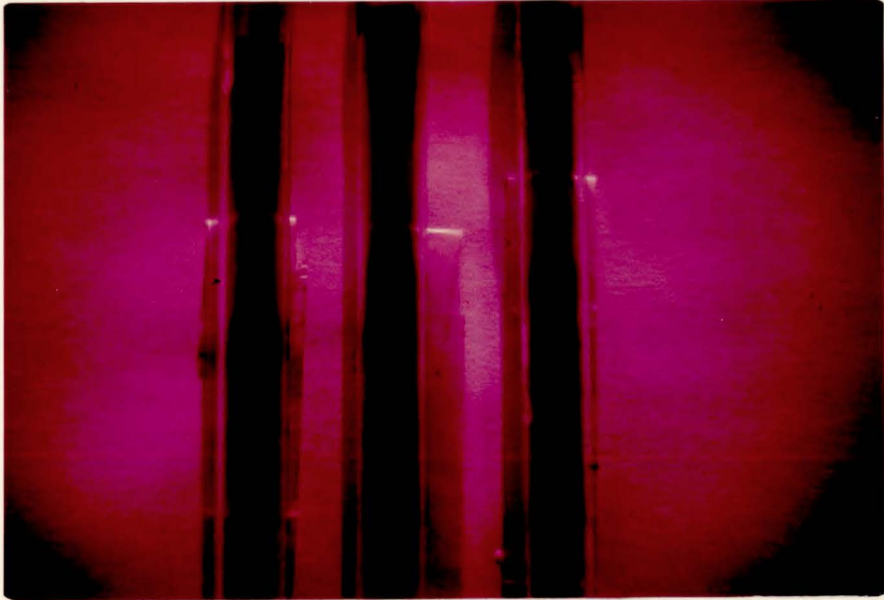


Figure 13. Sodium Dodecyl Sulfate Polyacrylamide Gel Electrophoresis of Sepharose CL-4B Purified High Molecular Weight Proteinase From Bovine Spleen.

Left gel: Molecular weight markers from the top; Thyroglobulin 330,000, Half Ferritin 220,000, Albumin 67,000, Catalase 60,000 and Lactate Dehydrogenase 36,000. Middle and Right: Sepharose CL-4B purified enzyme with the middle gel containing twice the sample load as the gel on the right. The very dark band corresponds to the bottom of the gel. Conditions for electrophoresis are described in the text.

Degradation of Beta-Insulin by the High Molecular Weight Proteinase and Separation of Peptides by Cation Exchange Chromatography

The beta-chain of Insulin was subjected to degradation by the Sepharose CL-4B purified enzyme from bovine spleen as described in methods. After addition of perchloric acid to a concentration of 1 M, the precipitate was cleared by centrifugation and the supernate placed on a 1.6 x 29 cm column of Sephadex G-25SF equilibrated in 0.05 M monobasic sodium phosphate. The A_{210} elution from this column is shown in Figure 14. Fractions 24-33 were pooled and placed on a column of Bio-Rad AG50W-X2 cation exchange resin of 0.9 x 60 cm. The column was equilibrated in pyridine-acetate buffer pH 3.1 and elution was by a gradient to pH 5.0 in pyridine-acetate. Ninhydrin determinations were performed on collected fractions with results showing elution of one major ninhydrin positive peak. Fractions containing this peak were dried under nitrogen and submitted to another laboratory for automated amino acid analysis. Results were consistent with the amino terminal 15 amino acids of the beta-chain of Insulin which is illustrated in Figure 15.

Fractions 35-42 from the Sephadex G-25SF column were pooled and separated into 4 ninhydrin positive peaks by cation exchange chromatography. Each peak was submitted for amino acid analysis with only trace levels of amino

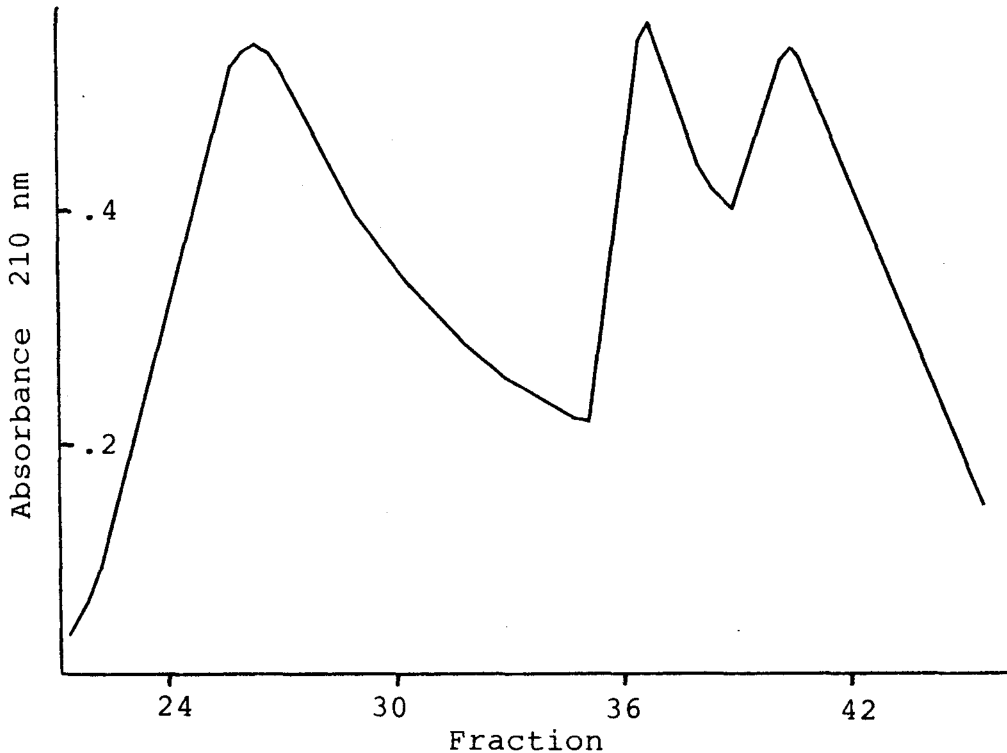


Figure 14. Elution From Sephadex G-25SF of Perchlorate Soluble Peptides.

Peptides resulted from reaction of Sepharose CL-4B purified high molecular weight proteinase from bovine spleen with the beta-chain of Insulin. Conditions of chromatography are described in the text.

Phe-Val-Asn-Gln-His-Leu-Cys-Gly-Ser-His-

Leu-Val-Glu-Ala-Leu-Tyr-Leu-Val-Cys-Gly-

Glu-Arg-Gly-Phe-Phe-Tyr-Thr-Pro-Lys-Ala

Figure 15. The Amino Acid Sequence of the Beta-Chain of Insulin

acids being detected. Thus, it was not possible to correlate these peaks with the amino acids or sequence of the beta-chain of Insulin.

Degradation of Beta-Insulin by the High Molecular Weight Proteinase and Separation of Peptides by High Performance Liquid Chromatography

The beta-chain of Insulin was subjected to degradation by the enzyme as described in methods. Aliquotes were removed from the assay at timed intervals and peptides separated by C_{18} reversed phase high performance liquid chromatography with eluant peaks monitored at 214 nm. Column elutions showed the appearance of a number of distinct peaks which increased in size as the time of incubation of the assay increased. Furthermore, the peak identified as the beta-Insulin peak decreased in size with increasing assay time. This data is shown in Table 3. Appropriate blanks were run to rule out any contribution from either the incubated enzyme or substrate.

Substrate concentrations were determined by comparing peak height to a linear standard plot of beta-Insulin peak height versus concentration of injected sample. A **reciprocal** plot of rate data for the degradation of the beta-chain of Insulin was prepared and is shown in Figure 16.

Similar assays were run with dithiothreitol

Table 3. APPEARANCE OF PEPTIDES DERIVED FROM BETA-INSULIN DEGRADATION BY THE HIGH MOLECULAR WEIGHT PROTEINASE FROM BOVINE SPLEEN

Data is expressed as peak height at 214 nm as determined by C_{18} reversed phase high performance liquid chromatography. Peak "I" is the undegraded beta-chain of Insulin. Peaks "A-H" do not appear in the chromatograms when either the beta-chain of Insulin or the enzyme are incubated separately. Peaks "A-H" showed an increase in absorbance of each peak which paralleled a decrease in absorbance in peak "I", suggesting that none of these peaks were related to each other as precursors to products.

Minutes of Incubation	Peak Identification								
	A	B	C	D	E	F	G	H	I
0	-	-	-	-	-	.041	.052	.054	1.097
15	.005	.001	.024	.028	.027	.049	.110	.054	1.093
30	.008	.003	.030	.047	.043	.076	.147	.070	1.035
45	.011	.007	.043	.068	.069	.099	.192	.086	.881
60	.012	.009	.051	.090	.092	.120	.216	.095	.804
90	.018	.017	.069	.134	.149	.172	.240	.114	.757
120	.025	.024	.088	.175	.201	.197	.266	.121	.659
180	.037	.036	.143	.283	.262	.280	.298	.136	.546
240	.046	.044	.188	.359	.374	.345	.356	.124	.350
300	.057	.052	.260	.518	.400	.345	.308	.127	.173

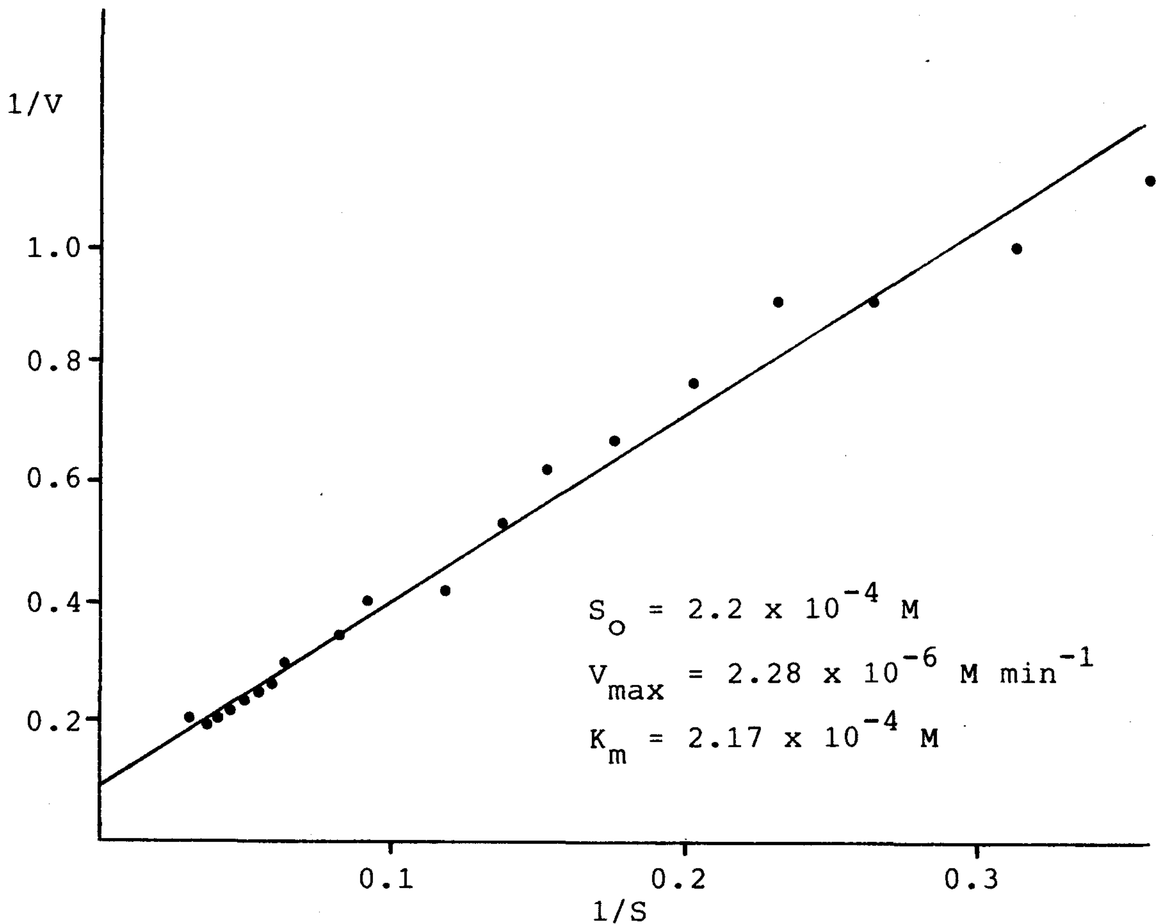


Figure 16. Reciprocal Plot of Rate Data for the Degradation of the Beta-Chain of Insulin by the High Molecular Weight Proteinase from Bovine Spleen.

Reaction rates were calculated from slopes obtained from a plot of substrate concentration versus time. Substrate concentrations at each time point were obtained by determining the height of the beta-Insulin peak using high performance liquid chromatography.

omitted from the incubation mixture. Quantitation of the beta-Insulin concentration remaining at various incubation times showed a marked decrease in the rate of disappearance of substrate up to 100 minutes of incubation. After 120 minutes however, the rate of disappearance of the substrate approached that obtained when dithiothreitol was included in the assay. The rate of disappearance of beta-Insulin in the presence and absence of dithiothreitol is shown in Figure 17.

pH Dependency in Acetate Buffers Using the Beta-Chain of Insulin as Substrate

Beta-Insulin in 0.5 M acetate buffers over a range of pH was included in assay mixtures containing final concentrations of 5 mM dithiothreitol, 2.5 mM EDTA, 10^{-5} M beta-Insulin and approximately 2 micrograms of enzyme protein. Reaction mixtures were incubated at 37°C for 40 minutes and the reaction stopped by freezing in dry ice-acetone. Quantitation of beta-Insulin remaining at various times of incubation was by high performance liquid chromatography as previously described. Results in Figure 18 show a pH optimum of approximately 3.9.

Inhibitors of Proteolytic Enzymes

Effects of various inhibitors were studied using the beta-Insulin assay and quantitation of remaining

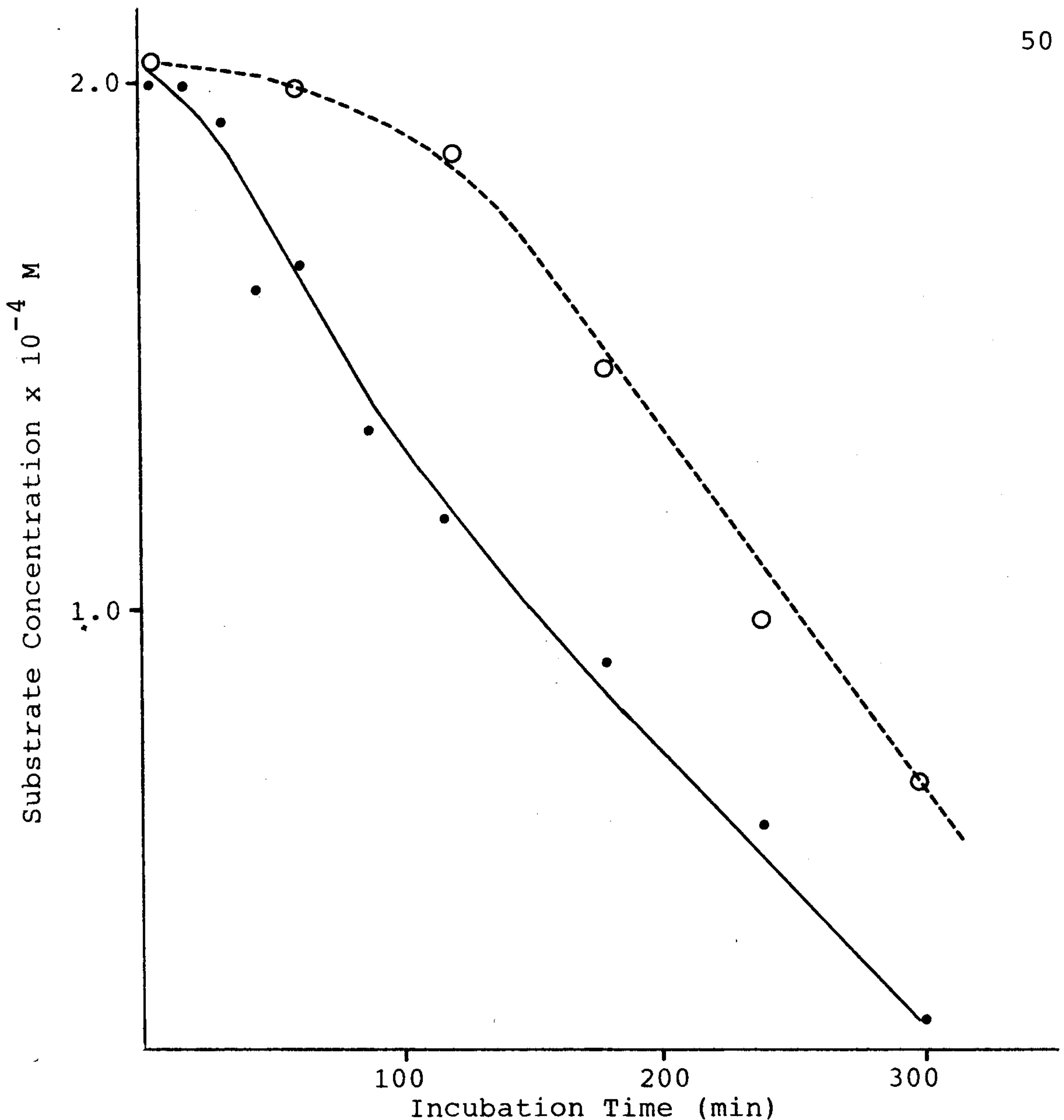


Figure 17. Disappearance of the Beta-Chain of Insulin When Incubated with the High Molecular Weight Proteinase from Bovine Spleen in the Presence and Absence of Dithiothreitol.

Substrate concentrations at each time point were obtained by determining the peak height using high performance liquid chromatography. (——) Assay with dithiothreitol. (-----) Assay with water in place of dithiothreitol. Conditions of chromatography and enzyme assay are described in the text.

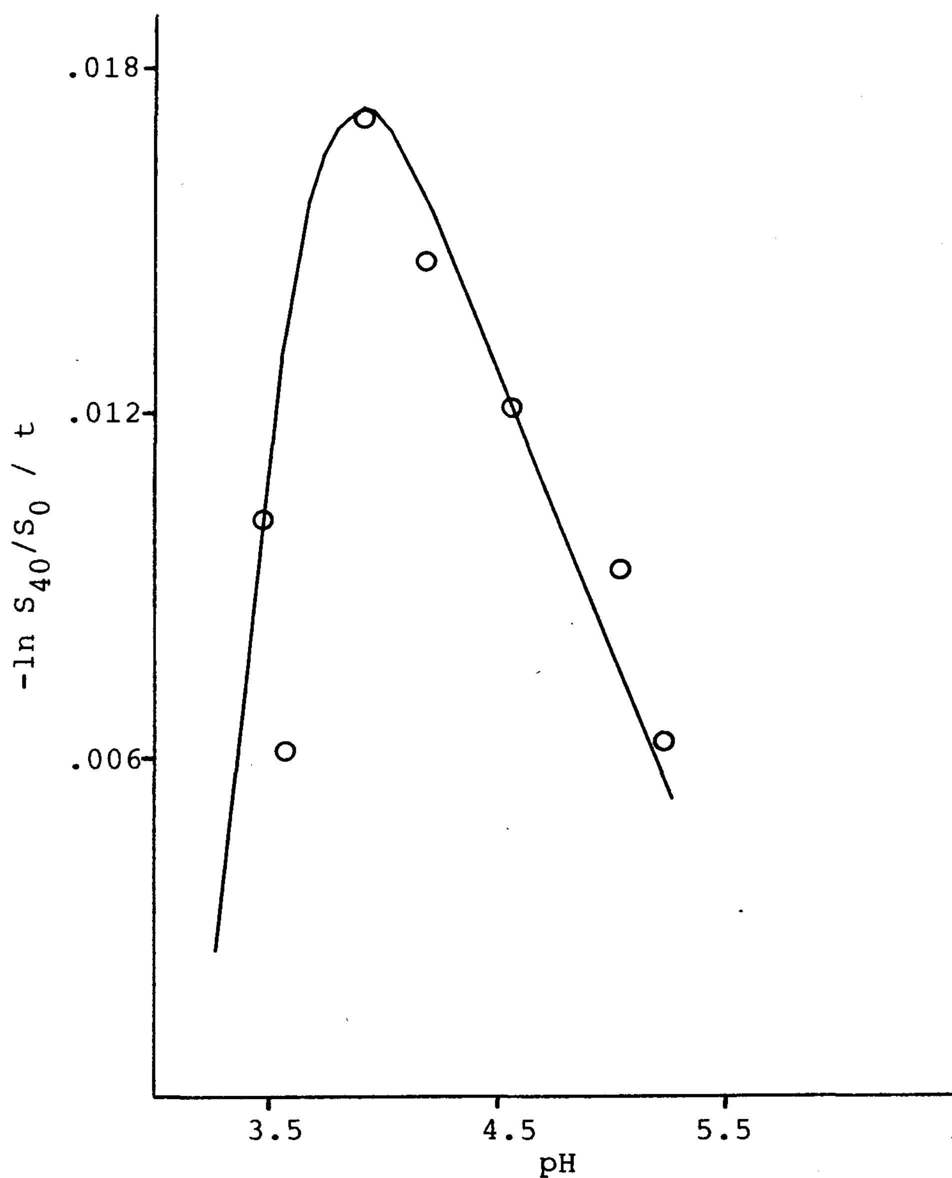


Figure 18. The pH Dependency of the High Molecular Weight Proteinase from Bovine Spleen.

Assays were performed in 0.5 M sodium acetate buffer at the indicated pH, using the beta-chain of Insulin as substrate. Conditions of enzyme assay and substrate quantitation are described in the text.

substrate using high performance liquid chromatography as described in methods. Results shown in Table 4 indicate the percent inhibition of beta-Insulin hydrolysis at various final inhibitor concentrations in the assay mixture as compared to control assays without the inhibitor included. The enzyme was preincubated with inhibitor at six times the listed concentration for 15 minutes at room temperature before activation with dithiothreitol.

Assays which included Pepstatin as the inhibitor at a final concentration of 0.5 mM showed no inhibition of degradation of the substrate. Efforts to study higher concentrations of Pepstatin were hindered by lack of solubility of the inhibitor in the assay mixture.

Degradation of Glycylglycyl-L-phenylalanyl-L-phenylalanine Ethyl Ester

The rate of disappearance of this substrate with time is shown in Figure 19. Substrate concentrations in assay aliquotes taken after various times of incubation, were determined by high performance liquid chromatography as described in methods.

The rate of appearance of a new peak eluting at a point earlier in the gradient than the substrate is shown in Figure 20. Incubated substrate and enzyme blanks were also run to rule out the appearance of the new peak from these sources.

Table 4. EFFECTS OF VARIOUS PROTEINASE INHIBITORS ON THE ACTIVITY OF THE HIGH MOLECULAR WEIGHT PROTEINASE FROM BOVINE SPLEEN AGAINST THE BETA-CHAIN OF INSULIN.

[a]Assay time in minutes.
[b]Data expressed as the beta-chain of Insulin peak height at 214 nm on high performance liquid chromatography.
[c]Inhibitor concentrations listed are final concentrations in the assay mixture. Percentages in parentheses indicate the percent reduction in activity as compared to the control assay in Table 3. Percent reduction was calculated by comparing the difference in peak heights at time zero and the indicated times for the control, with those differences obtained in the corresponding inhibited reaction.

	Assay Time[a]	Control[b]	1 mM[c]	10 mM[c]
ANTIPAIN	0	1.097	.752	1.169
	90	.757	----	----
	180	.546	.408(27%)	1.013(73%)
	222	.408	----	----
	277	.240	----	----
	300	.173	.240(37%)	.902(73%)

	Assay Time	Control	1 mM	10 mM
LEUPEPTIN	0	1.097	1.129	1.165
	180	.546	.722(18%)	1.022(75%)
	300	.173	----	.813(65%)

	Assay Time	Control	0.3 mM	30 mM
DIAZOACETYL NORLEUCINE METHYL ESTER	0	1.097	.359	.344
	240	.350	----	----
	288	.220	----	----
	300	.173	.020(0%)	.220(85%)

	Assay Time	Control	1% Solution
HYDROGEN PEROXIDE	0	1.097	.222
	180	.546	.183(95%)
	288	.220	----
	296	.183	----

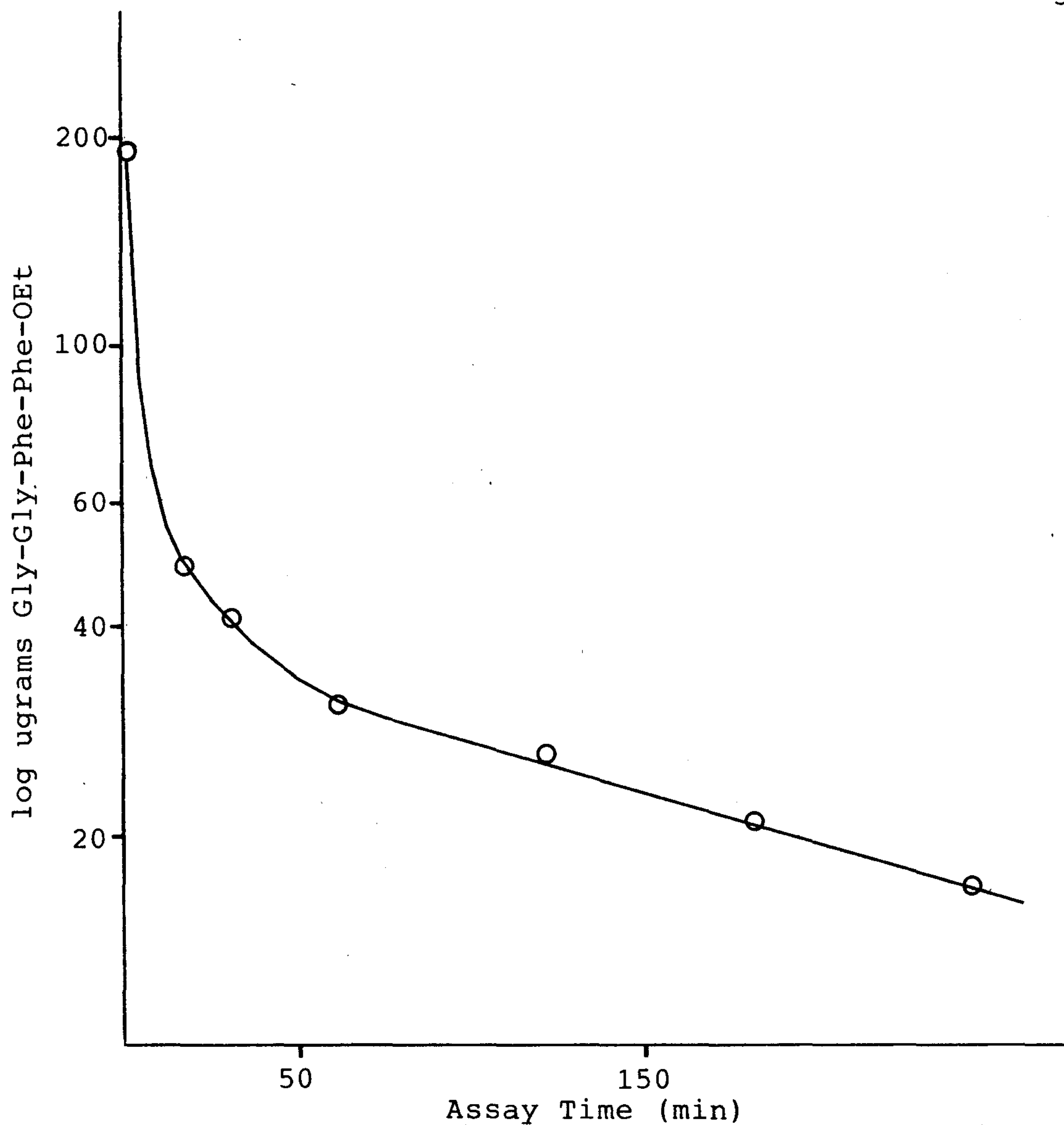


Figure 19. Rate of Degradation of Glycylglycyl-L-phenylalanyl-L-phenylalanine Ethyl Ester (Gly-Gly-Phe-Phe-OEt) by the High Molecular Weight Proteinase from Bovine Spleen.

The concentration of substrate remaining at each time point was determined by high performance liquid chromatography. Conditions of chromatography are described in the text.

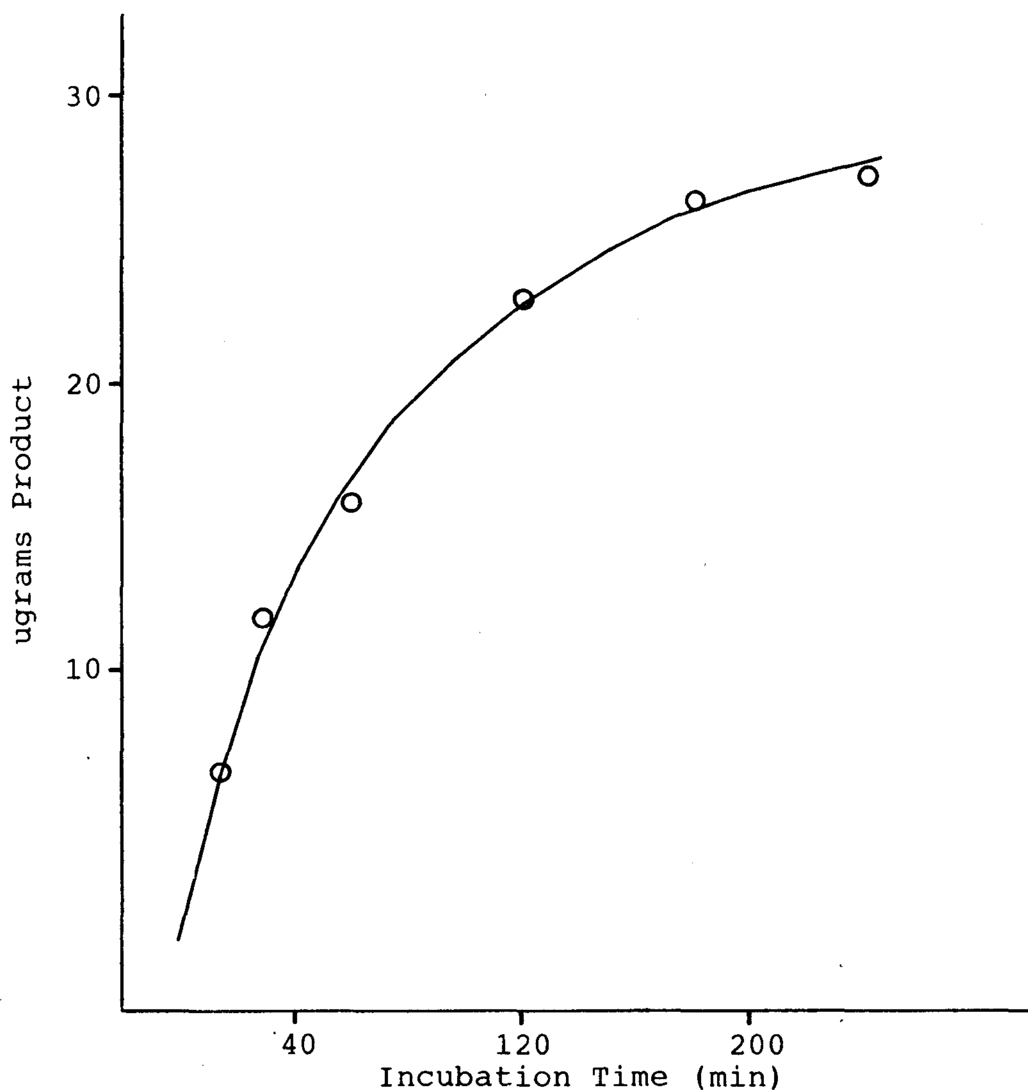


Figure 20. Rate of Appearance of Product Formed During Degradation of Glycylglycyl-L-phenylalanyl-L-phenylalanine Ethyl Ester by the High Molecular Weight Proteinase from Bovine Spleen.

The new peak eluted at 23 minutes on the gradient compared to 39 minutes for the native substrate. Micrograms of product were calculated based on the molecular weight of phenylalanylphenylalanine ethyl ester.

Glycylglycyl-L-phenylalanine and Phenylalanine Ethyl Ester were subjected to high performance liquid chromatography in an identical manner. These compounds eluted at points in the gradient much earlier than either the substrate or the new peak described in Figure 20. This suggests that the new peak may be either Phenylalanyl-L-phenylalanine Ethyl Ester or Glycyl-L-phenylalanyl-L-phenylalanine Ethyl Ester with the substrate being cleaved either between the glycine and phenylalanine residues or between the two glycine residues.

Effect of Adenosine Triphosphate and Magnesium on the Degradation of Beta-Insulin

Results shown in Table 5 indicate the effect of various inclusions on the degradation of beta-Insulin, for 30 minutes at 37°C and pH 6.0, by the high molecular weight proteinase from bovine spleen. A marked increase in beta-Insulin degradation is observed by addition of ATP and magnesium to an assay mixture already activated by dithiothreitol. This same increase in activity apparently mediated by ATP was not observed at all in assays carried out in formate buffers at pH 3.5 under otherwise identical conditions.

Table 5. EFFECT OF ATP AND MAGNESIUM ON THE ACTIVITY OF THE HIGH MOLECULAR WEIGHT PROTEINASE FROM BOVINE SPLEEN AT pH 6.0

DTT (8mM)	ATP (5mM)	Mg (5mM)	% Substrate Degraded
---	---	---	15
---	---	+++	4
---	+++	+++	30
+++	---	---	35
+++	---	+++	35
+++	+++	---	45
+++	+++	+++	70

ATP, adenosine triphosphate; DTT, dithiothreitol; Mg, added as magnesium chloride. (+++) indicates inclusion in the assay. (---) indicates omission from the assay. Beta-Insulin substrate was quantitated by high performance liquid chromatography. Conditions of enzyme assay are described in methods.

CHAPTER IV

DISCUSSION

Data for recovery and purification of mercury sensitive Hemoglobin Hydrolase activity through the various steps of purification indicate a purification of 20-40 fold and a yield of 2-4% in various spleen workups. The crudeness of the pH 6.1 supernatant at the first purification step and the large amount of protein present, made it difficult to do a correction for the A_{280} contribution of the blank to the hemoglobin assay of this fraction. For this reason, purification and yield data were calculated based on the pH 4.4 supernatant in the following purification step. Calculation of the recovered activity throughout the purification and isolation procedures indicated that a large portion of the total Hemoglobin Hydrolase activity in bovine spleen homogenate was due to the high molecular weight sulfhydryl dependent proteinase and this enzyme accounted for approximately 50% of the mercury sensitive Hemoglobin Hydrolase activity at pH 3.4. These proportions were similarly observed in homogenates of whole rat liver.

Using the technique of differential ultracentrifugation on homogenates of perfused rat liver, mercury

sensitive Hemoglobin Hydrolase activity at pH 3.4 was shown to have a light mitochondrial, or lysosomal, distribution and this activity eluted from a Sephadex G-200 column at the same high apparent molecular weight as that activity found in whole organ homogenates. While the technique of differential ultracentrifugation by no means yields a homogeneous preparation of lysosomes, the determination of a pH optimum of 3.4 for the degradation of hemoglobin by this enzyme is consistent with the experimentally determined subcellular distribution pattern of the activity.

The native protein eluted from a calibrated column of Sepharose CL-4B at an apparent molecular weight of 395,000 daltons. While polyacrylamide gel electrophoresis and thin layer isoelectric focusing of Sepharose CL-4B purified enzyme yielded only single bands of protein, SDS polyacrylamide gel electrophoresis yielded three distinct protein bands of 95,000, 60,000 and 40,000 daltons. Much stronger dissociating conditions were necessary than the 1% SDS and 1% mercaptoethanol in the pretreatment buffer used by Weber (1972). Despite the use of 4% acrylamide gels, it was necessary to use 5% SDS and 2% mercaptoethanol in the pretreatment buffer in order to get the protein to penetrate the gels during electrophoresis. In another experiment, a Sephadex G-200 column equilibrated in 50 mM acetate buffer pH 5.3 containing 0.03 M cysteine

failed to elute the enzyme at a lower apparent molecular weight than the column not containing cysteine.

When the enzyme was incubated with the pepsin substrate Glycylglycyl-L-phenylalanyl-L-phenylalanine Ethyl Ester, high performance liquid chromatography of reaction aliquotes showed the appearance of a reaction product that suggestive evidence indicates may be Phenylalanylphenylalanine Ethyl Ester. Also, earlier experiments had recovered the amino terminal fifteen amino acids from the beta-chain of Insulin when assay degradation products were separated by cation exchange chromatography and analyzed for amino acid content. In both of these instances, cleavage at the amino end of a hydrophobic residue is apparently indicated.

High performance liquid chromatography also provided a useful tool to monitor the disappearance of the beta-chain of Insulin when incubated with the enzyme and at the same time to monitor the appearance of a limited number of discrete peaks corresponding to degradation products. This highly reproducible appearance of a relatively small number of peaks suggests a well defined specificity for this enzyme. This specificity may indeed include or be limited to cleavage at the amino end of hydrophobic residues.

One experimental observation makes it difficult to assign a specific role to dithiothreitol in the

activation of the enzyme. Degradation of the beta-chain of Insulin occurred at a greatly reduced rate for the first 120 minutes of the assay when dithiothreitol was omitted, but the rate subsequently increased to equal the rate of the assay which included dithiothreitol. Since the beta-chain of Insulin used as a substrate contained only cysteic acid residues, the apparent slow activation in the absence of dithiothreitol was not due to formation of free cysteine in the medium.

Of the functional groups in proteins, the sulfhydryl group is usually the most reactive toward nucleophilic reagents, and in the sulfhydryl enzymes, a particular sulfhydryl group is often more reactive than others (Liu, 1977). The reactivity of a given sulfhydryl group to dithiothreitol acting as a nucleophile may be modified by adjustment of reaction conditions, by prior alteration of other protein groups, or by denaturration. The factors which determine sulfhydryl group reactivity include location in the three dimensional structure of the protein, neighboring group effects and interaction with other functional groups. In addition, the strong affinity of some heavy metals to bind to sulfhydryl groups may also suggest an activation role for dithiothreitol as a chelator. These considerations may be pertinent to the interpretation of enzyme activity changes in the presence

of dithiothreitol.

Tissue proteinases have been classified as thiol, carboxyl, serine, or metalloproteinases based on the class of catalytic mechanism, and since inhibitors are useful in determining the nature of the catalytic group, they are an important tool in classifying the enzyme. Experiments with various inhibitors of proteolytic enzymes using the beta-chain of Insulin as substrate indicated that inhibitor concentrations in the range of 10 mM were necessary to show significant enzyme inhibition. This contrasts sharply with data reported by Barrett (1973) which showed complete inhibition of the thiol proteinase Cathepsin B by 1 uM Leupeptin. In addition, Smith (1969) has reported inhibition of Cathepsin D, a carboxyl proteinase, by Diazoacetyl-DL-norleucine Methyl Ester at equimolar ratios and Barrett and Dingle (1971) have reported complete inhibition of Cathepsin D by 1 uM Pepstatin.

Although experiments using Leupeptin and Antipain at 1 mM and 10 mM concentrations may show a proportionate increase in inhibition, there is no sign of stoiciometric inhibition as has been reported for the inhibition of Cathepsin D by Diazoacetyl-DL-norleucine Methyl Ester. Further, the high concentrations of Leupeptin and Antipain required, seem to suggest that the observed inhibition may be simple competitive inhibition and not inhibition

by formation of a transition state analog.

Studies of the rate of disappearance of the beta-chain of Insulin in the presence of dithiothreitol (DTT), adenosine triphosphate (ATP) and magnesium showed that ATP and magnesium double the rate of degradation of the substrate in acetate buffer pH 6.0 compared to assays including only DTT. Neither ATP nor magnesium alone showed this enhancement of enzyme activity. Further, this enhancement of enzyme activity was not observed at all in assays buffered in acetate at pH 3.5. This observation is of note since the pH optimum for the degradation of the beta-chain of Insulin in acetate buffer and in the presence of DTT was determined to at pH 3.9.

This change in enzymic activity mediated by ATP may be caused by an alteration in the substrate binding site of the enzyme whereby the pK_a of the active site residues would be changed. Pillai (1983) however, has reported that ATP does not alter the ability of Cathepsin D to bind Pepstatin. The role of magnesium in mediating the activation by ATP is unclear. The presence of a metalloproteinase can be ruled out since the enzymic activity is not inhibited by EDTA. Further, compared to assay with only DTT present, ATP alone increases activity by 30% but magnesium alone shows no effect.

Within the cytoplasm of mammalian cells, there is

a soluble ATP dependent proteolytic system that selectively degrades polypeptides with highly abnormal structures (Hershko, et al, 1978) as well as short lived normal enzymes (Grinde and Jahnsen, 1982), and is probably the major pathway for protein breakdown in reticulocytes as they mature into erythrocytes (Boches and Goldberg, 1982). Hershko, et al, (1979) resolved the ATP-dependent proteolytic system from reticulocytes into three components, each of which was absolutely required for acid solubilization of bovine serum albumin at pH 7.6, and found one of these components, a serine protease of approximately 450,000 daltons, to interact directly with ATP. Kirschner and Goldberg (1983) while further isolating this same enzyme from red cell lysates, described a metalloproteinase with an apparent molecular weight of 300,000 daltons and composed of 115,000 dalton subunits which hydrolyzed Insulin at pH 8.5. He suggested that this enzyme may catalyze subsequent steps after initial cleavage by the larger enzyme.

Watabe, et al, (1979) showed that polyphosphates, including ATP, can directly increase Cathepsin D proteolysis. Pillai, et al, (1983) observed that the activation also occurs with nonhydrolyzable ATP analogs and also suggested the possibility that ATP may activate lysosomal proteolysis directly and that hydrolysis of the nucleotide

triphosphate is not required for such activation.

Intracellular proteases serve a number of physiologically important functions. For example, membrane associated enzymes are important in the processing of secreted proteins (Kenny, 1977), while lysosomal cathepsins appear responsible for degrading pinocytized and membrane proteins (Lloyd, 1980) and for the accelerated degradation of intracellular proteins during starvation (Dean, 1980). Cytosolic endopeptidases with pH optima in the range of 7.0-8.5 have been described which probably play a role in the extralysosomal pathway of intracellular protein degradation (Grinde and Seglen, 1980). Recently, other cytoplasmic proteases have been purified from mammalian cells and found to be large and multimeric (Barrett, 1977; Boches, et al, 1980; Goldberg, et al, 1981; Pontremoli, et al, 1980; Gade and Brown, 1978). In addition, cysteine proteases of 230,000 and 650,000 daltons have recently been described in liver particulate fractions (Liao and Lenney, 1984 and 1985).

Wilk and Orlowski (1980) reported the isolation of a highly purified cation sensitive neutral endopeptidase from bovine pituitaries with an apparent molecular weight of about 700,000 daltons. This enzyme was found to split peptide bonds on the carboxyl side of hydrophobic amino acids (chymotrypsin-like activity), basic amino

acids (trypsin-like activity),and acidic amino acids (peptidyl-glutamyl-peptide bond hydrolyzing activity) (Wilk and Orlowski, 1983). Experiments with inhibitors and activators indicated the three activities were catalyzed by distinct components, with the trypsin-like component being inhibited by leupeptin and activated by thiols. The enzyme further showed concentration dependent inhibition of all three activities by cations such as Na^+ and K^+. Dissociation of the enzyme led to loss of all catalytic activities, indicating that the integrity of the macromolecule was essential for expression of the three activities.

Dahlmann (1985) described three high molecular weight cysteine proteinases in rat skeletal muscle, each having a molecular weight in the range of 650,000-750,000 daltons. One of these enzymes was termed a 'multicatalytic proteinase' since it hydrolyzed N-blocked tripeptide substrates with an arginine, phenylalanine or glutamic acid residue adjacent to the leaving group. Additionally, the enzyme contained caseinolytic activity and also degraded peptides such as insulin and neurotensin. Multicatalytic proteinase activities toward tripeptide substrates and against casein had different pH optima in the basic range and were affected by leupeptin, chymostatin and calcium to varying extents or even in an opposite fashion

(Dahlmann, et al, 1985a). Activities toward tripeptide substrates were increased 7-14 fold by SDS and 50 fold by oleic acid (Dahlmann, et al, 1985b). In contrast to the non-activated proteinase, the activated enzyme considerably degraded muscle cytoplasmic proteins in vitro. This multicatalytic proteinase may indeed be involved in the calcium dependent proteolytic pathway involving thiol proteases in rat muscle described by Baracos and co-workers (1986).

A high molecular weight neutral protease from skeletal muscle has also been described (Ishiura, et al, 1985). This protease, termed ingensin, had a molecular weight of above 1,000,000 daltons and was isolated in two forms having different sensitivities to monovalent and divalent cations, protease inhibitors and to activation by SDS. One form of ingensin was later isolated from reticulocyte lysate and found to account for more than 90% of the casein degrading activity in reticulocyte extracts (Ishiura, et al, 1986). The enzyme was inhibited by inhibitors of ATP-dependent proteolysis , suggesting that ingensin is involved in ATP-dependent degradation of casein.

Additional high molecular weight proteases from liver cytosol have been described by Rivett (1985). Two of these were calcium dependent thiol proteases having molecular weights of 150,000 and 200,000 daltons, and

differing in their calcium requirement. A third was a calcium independent protease having a molecular weight of 300,000 daltons which degraded an oxidized form of glutamine synthetase at pH 8.0.

Also of high apparent molecular weight, the enzyme described in this thesis has a native molecular weight of approximately 395,000 daltons and is composed of subunits of 95,000, 60,000 and 40,000 daltons. Based on these and its other properties, this enzyme is unique when compared to any known mammalian cytosolic or lysosomal proteases.

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APPROVAL SHEET

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The thesis is therefore accepted in partial fulfillment of the requirements for the degree of Master of Science.

March 26, 1987
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