STUDIES ON PHYSICO-CHEMICAL AND KINETIC CHARACTERIZATION OF CATHEPSIN B FROM GOAT SPLEEN

ABSTRACT

BY

SANTANU DEB CHOUDHURY

THESIS
SUBMITTED IN FULFILMENT OF THE REQUIREMENTS
FOR THE DEGREE OF
DOCTOR OF PHILOSOPHY IN BIOCHEMISTRY

TO

NORTH-EASTERN HILL UNIVERSITY
SHILLONG - 793 022
INDIA

1995

ABSTRACT

Considerable research on lysosomal proteolytic enzymes over the past few decades have emphasized their wide range participation in vital physiological and pathological processes. The wide distribution of lysosomal cathepsins, a group of proteolytic enzymes, throughout most tissues, suggest that they play an important role in cellular protein turnover. Mammalian tissues are known to contain several proteinases with similar and overlapping enzymatic properties. Availability of specific substrates and inhibitors has helped in identification of more than a dozen cathepsins and related proteinases . Proteinases of lysosomal origin have been categorized into four groups, namely, cysteine, aspartic, serine and metalloproteinases. The classification is based on the nature of the most prominent functional group in the active site of the enzyme, which in the case of cysteine proteinases is an essential cysteine residue. These include cathepsins B, H, L and S.

One of the most thoroughly studied thiol proteinases, cathepsin B (EC 3.4.22.1) is essentially a carboxypeptidase which releases dipeptides sequentially from the carboxyl termini of polypeptides. However, it also exhibits endopeptidase activity with a specificity towards an arginyl-arginyl-x bond.

Apart from its involvement in various vital physiological functions such as protein turnover and processing of proteins and hormone precursors, it has been implicated in various diseases and has been reported to play significant role in tumour growth, invasiveness and metastasis.

The outcome of research on cathepsin B particularly of its isozymes, have not been unequivocal. While the enzyme was found to be homogeneous on polyacrylamide gels from some tissues, multiplicity of bands of cathepsin B preparations from other tissues have been attributed to microheterogeneity representing some related proteins and/or degradation products of the active enzyme. However the earlier reports on isozymes of cathepsin B with isoelectric pHs and molecular weights in the range of

4.5-5.5 and 24-29 kDa, respectively and our present study from goat spleen indicate that this tissue is histologically heterogeneous consisting of many different cell types with varying degrees of differentiation which may account for enzyme species with different maturational states.

In the present study goat spleen was chosen as a source and a simple procedure was developed for the isolation and purification of cathepsin B. A striking feature of the goat spleen cathepsin B is its elution by CM-Sephadex ion exchange chromatography at a relatively lower pH and ionic strength. The enzyme was eluted at pH 5.6 as against pH 6.0 and 0.1 M NaCl, required for a similar elution of cathepsin B from porcine spleen, which may be attributed to differences in the nature and extent of glycosylation and/or amino acid composition of the enzymes from the two sources.

The purified cathepsin B from goat spleen yielded two distinct protein bands on PAGE. It was therefore subjected to affinity chromatography on ConA-Sepharose 4B column. Two distinct and enzymatically active protein peaks were obtained which were named goat spleen cathepsin B-I (GSCB-I) and goat spleen cathepsin B-II (GSCB-II). GSCB-I, which did not bind to ConA-Sepharose column possibly due to differential glycosylation of the asparagine residues, constituted approximately 84% of the total enzyme activity and had a specific activity of 4.16 units/mg protein towards Z-Arg-Arg-MCA, where as GSCB-II, which was eluted with 0.5 M α-D-glucopyranoside constituted about 16% of the total activity and had a specific activity of 1.06 units/mg protein. Molecular weights of GSCB-I and GSCB-II determined by gel filtration were 28.1 kDa and 31.6 kDa, respectively and by SDS-PAGE, yielded a value of 25.7 kDa and 26.6 kDa respectively. Both enzyme fractions lacked multiple chain forms. The Stokes radius calculated for GSCB-I and II were 2.46 Å and 2.58 Å with a frictional ratio of 1.22 and 1.28 respectively, suggesting globular conformations of the enzymes under native conditions.

The experiments in this study were performed with GSCB-I (unless stated otherwise) since it was present in larger amounts and had a much higher specific activity compared to GSCB-II.

GSCB-I was found to be a glycoprotein having a carbohydrate content of 4.6%. The intrinsic viscosity of the enzyme was determined to be 3.30 ml/gm, which was well within the range (3.0-4.0 ml/gm) expected for native proteins having compact and globular conformations. NH_2- and COOH- terminal amino acid residues were found to be Leu and Thr, respectively. A total of 0.9 and 1.6 mols of thiol groups could be titrated per mol of protein in absence and presence of 8 M urea respectively, clearly showing that the free thiol group(s) in the enzyme is/are not fully exposed.

Amino acid composition of GSCB-I showed close similarities with cathepsin B from rat, bovine, human and porcine tissues, except for Ser, the amounts of which were relatively lower. Tyr and Lys were also significantly lower whereas Leu and Trp were present at relatively higher amounts. U.V. absorption spectra of the enzyme showed a maxima at 278 nm. The fluorescence emission maxima was near 340 nm which is characteristic of proteins containing significant amount of Trp residues. The specific extinction coefficient ($E^{1\%}_{1cm}$) of the enzyme was determined to be 15.64. GSCB-I had an isoionic pH of 5.12 indicating an acidic nature. This value is consistent with the values of pH range 4.8-5.3 for cathepsin B from different sources.

Thiol reducing agents had a strong stimulatory effect on catheptic activity. Among the thiol modulators tested, cysteamine was most effective and thioglycerol was least effective. These results indicate differential effect of thiol group modulators in altering the activity of this enzyme.

Influences of various inhibitors on GSCB-I show divalent cations such as Mn^{++} and Hg^{++}, alkylating agents like iodoacetic acid and iodoacetamide and peptidyl inhibitors such as E-64, antipain and leupeptin inactivated the enzyme. Maleic and succinic anhydride used for modification of α-amino group of proteins showed very mild inhibitory effect. Pepstatin, a well known inhibitor of aspartyl proteinases, including cathepsin D, had no inhibitory effect on enzyme activity. Indomethacin, an anti-inflammatory agent, caused about 38% inhibition of the enzyme activity. This is in

accordance with 37% inhibition of rat spleen cathepsin B activity, which has been shown to be due to a conformational change brought about by indomethacin on the enzyme. This may be a contributory factor in reducing inflammation by lowering the rate of proteolysis.

GSCB-I was markedly inhibited by Gdn-HCl and urea. 50% inactivation of the enzyme was achieved at a urea concentration of about 0.15 M and virtually no activity was found above 1.0 M. The process of inactivation became irreversible as the urea concentration was raised beyond 2.5 M. This also ruled out the possible contamination of the cathepsin B preparation with cathepsin L as cathepsin L activity is retained even at a urea concentration of 3 M. Gdn-HCl was more effective against the enzyme and 50% inactivation was achieved at 0.12 M. The loss of catheptic activity was reversible at and below 1.8 M Gdn-HCl, but above 2.0 M concentration the inactivation process became irreversible. Since the concentrations of the denaturants are low to cause any significant conformational alterations of the enzyme, the loss of activity may be possibly due to perturbations near the environment of the enzyme active site. TPCK inhibition of the enzyme at 0.01 mM concentration was about 53% and the enzyme was fully inactivated at a TPCK concentration of 0.1 mM and above, indicating that cathepsin B activity may be similar to chymotryptic activity in the mechanism of inhibition by TPCK.

GSCB-I was found to be very sensitive to physical parameters such as pH, temperature and ionic strength. The enzyme showed highest activity at a pH of 6.8 and was fully stable for at least 20 min upto pH 7.0. The enzyme was found to have maximum activity at physiological temperature and was fairly stable and retained most of its activity till the temperature was raised above 40°C. Maximum activity of the enzyme was observed at an ionic strength of 0.22. However at higher salt concentrations, a pronounced decrease in the activity was observed. Hence, 20 mM sodium phosphate buffer or other buffers of equivalent ionic strength were used for the assay of the enzyme activity.

The kinetic parameters K_m and V_{max} of GSCB-I showed that the enzyme had greater catalytic potential at acidic and/or near neutral pHs,

for various natural and synthetic substrates. Among the synthetic substrates tested Z-Phe-Arg-MCA with a K_m of 0.07 mM was found to be most preferred followed by Z-Arg-Arg-MCA (K_m=0.22 mM), BAPNA (K_m=0.70 mM) and BANA (K_m=2.64 mM). Hemoglobin, among the natural substrates tested for GSCB-I, proved to be the most preferred with a K_m of 1.46 μM followed by casein (K_m=2.18 μM) and BSA (K_m=2.67 μM). The ability of cathepsin B to inactivate aldolase have been shown to be a characteristic feature of this enzyme and it also differentiates cathepsin B from other cathepsins. However only about 20% aldolase inactivation was observed at aldolase to GSCB-I molar ratio of 50:1, which is significantly lower than the values reported for aldolase inactivation by cathepsin B from other sources.

A comparative study of GSCB-I and II showed that inhibitors such as leupeptin, antipain, pepstatin, iodoacetic acid and iodoacetamide and denaturants such as Gdn-HCl and urea showed close similarities in their mode of inhibition of both the enzyme fractions. However kinetic parameters of GSCB-II against the substrates Z-Phe-Arg-MCA, Z-Arg-Arg-MCA and BANA showed that though the enzyme was similar in its enzymatic activity against these substrates with that of GSCB-I, it differed significantly in its K_m and V_{max} as compared to that of GSCB-I. Among the substrates tested, Z-Phe-Arg-MCA was most preferred for GSCB-II (K_m = 0.05 mM) followed by Z-Arg-Arg-MCA (K_m=0.14 mM) and BANA (K_m=1.87 mM). No activity against the substrates Leu-NA or Arg-MCA were shown either by GSCB-I or II, thus ruling out the possibility of the presence of leucine aminopeptidases or cathepsin H in the enzyme preparations.

Immunological studies revealed that polyclonal antibodies raised in rabbits against the unresolved purified goat spleen cathepsin B (enzyme preparation before the ConA-Sepharose step) reacted only with purified goat spleen enzyme (unresolved goat spleen cathepsin B, GSCB-I and GSCB-II). However, no antigen-antibody reaction took place against buffalo kidney cathepsin B or cathepsin H from buffalo kidney or porcine lung.

A comparison of the results summarized above with that of the literature available on cathepsin B from other sources show that GSCB-I is similar to cathepsin B reported from other mammalian tissues with respect to most of the physicochemical properties. However, it differed significantly with regard to the number of some of its amino acid residues and catalytic efficiencies against various synthetic and natural protein substrates including muscle aldolase.

The differences, particularly in the catalytic efficiency and the possible presence of isozymes of goat spleen cathepsin B, reflect a species and/or tissue dependence of the enzyme.

STUDIES ON PHYSICO-CHEMICAL AND KINETIC CHARACTERIZATION OF CATHEPSIN B FROM GOAT SPLEEN

BY Choudhury

SANTANU DEB CHOUDHURY

THESIS
SUBMITTED IN FULFILMENT OF THE REQUIREMENTS
FOR THE DEGREE OF
DOCTOR OF PHILOSOPHY IN BIOCHEMISTRY

TO

NORTH-EASTERN HILL UNIVERSITY
SHILLONG - 793 022
INDIA
1995

पूर्वोत्तर पर्वतीय विश्वविद्यालय
पू० प० विवि० परिसर, शिलांग-७९३०२२ (मेघालय)
North-Eastern Hill University
NEHU Campus, Shillong-793022 (Meghalaya)

Phone :
Grams : NEHU

CERTIFICATE

We certify that the thesis entitled **"Studies on Physicochemical and Kinetic Characterization of Cathepsin B from Goat Spleen,"** submitted by **Mr. Santanu Deb Choudhury** for the degree of **Doctor of Philosophy** of the **North Eastern Hill University**, Shillong, embodies the record of original investigation carried out by him under our supervision. He has been duly registered and the thesis presented is worthy of being considered for the award of Ph. D. degree. This work has not been submitted for any degree of any other University.

(Ramesh Sharma)
Supervisor I

(M. Yahiya Khan)
Supervisor II

November 24th., 1995.

Forwarded

HEAD
Deptt. Biochemistry
NEHU, Shillong-22

ACKNOWLEDGEMENTS

I would like to convey to my supervisors Dr. R. Sharma, Head of Biochemistry, NEHU; and Prof. M.Y. Khan, presently on lien to the Deptt. of Biochemistry, Dr. R.M.L. University, Faizabad, a deep sense of gratitude for giving me an opportunity to work under them, for their constant guidance, support and encouragement throughout the course of my study.

I am also grateful to Prof. A.N. Rai, Dr. R.N. Sharan and Dr A. Alam for their valuable advice and suggestions rendered to me from time to time.

I am much obliged to Mr. P.K. Prabhakaran and Mr. D.K. Syiem for their help and for the long and useful discussions I had with them.

I would like to specially thank Dr. S.K. Agarwal for his constant help and support throughout. Thanks are also due to Dr. R. Sandhir, and to the members of the faculty of the Biochemistry Deptt., Dr. R.M.L. University, Faizabad.

I extend my special, heartfelt thanks to my co-workers, Mr. M. Lamsal, Mr. A.K. Patra, Ms. N. Choudhury, Mr. M.A. Borbhuiya, Mrs. S. Dey, Mr. L.S. Singh and Ms. B. Newar, all of whose help, co-operation and support has made this thesis possible.

I thank sincerely, my friends and colleagues, Dr. S. Ahmed, Dr. B.K. Das, Dr. M. Borthakur, Dr. B. Balachandran, Dr. K.K. Wary, Dr. R.S. Prakasham, Mr. S.K Bhuri, Mrs. S.Bhuri, Mr. S.K. Prasad, Mrs. J. Saikia, Ms. S. Chakraborty and Ms. C. Rapthap for their help and support.

I would like to extend my thanks to Mr B.K. Das, Mr. M.B. Singh and the staff of the Deptt. for the unconditional help rendered to me during my study period.

With a deep sense of gratitude, I thank my sister, wife and relatives for their inspiration, encouragement and affection given to me during my study tenure.

Last, but not the least, I thank NEHU for providing me with all infrastructural facilities and financial assistance in the forms of JRF and SRF during the period of research.

Santanu Deb Choudhury

24th November, 1995. Santanu Deb Choudhury

Dedicated to
my Parents

CONTENTS

LIST OF TABLES

LIST OF FIGURES

LIST OF ABBREVIATIONS

Arg-NA	Arginine-2-Napthylamide
Arg-MCA	L-arginine-7-amido-4-methylcoumarin
BANA	α-N-benzoyl-D, L-arginine-2-napthylamide
BAPNA	α-N-benzoyl-D, L-arginine-4-nitroanilide
BSA	Bovine serum albumin
BZ-Arg-NH_2	Benzoyl-arginine-amide
CM	Carboxymethyl
ConA	concanavalin A
Cbz-Phe-Ala-CNH_2	Benzyloxycarbonyl-(phenylalanyl-alanine)-diazomethane
DAB	p-dimethyl aminobenzaldehyde
DEAE	Diethylaminoethyl
DMSO	Dimethylsulfoxide
DTNB	5, 5'-dithiobis-(2-nitrobenzoic acid)
DTT	Dithiothreitol
EDTA	Ethylene diamine tetra acetic acid
E-64	trans-expoxy-succinyl-L-leucylamido (4-guanidino)-butane
FCA	Freund's complete adjuvent
Gdn-HCl	Guanidine hydrochloride
GSCB	Goat spleen cathepsin B
IFA	Incomplete Freund's adjuvent
Leu-NA	L-leucine-2-napthylamide
Leu-NH_2	L-Leucine amide
MCA	7-amino-4-methylcoumarin
PAGE	Polyacrylamide gel electorphoresis
PBS	Phosphate buffer saline
R_f	Relative front
R_m	Relative mobility
SDS	Sodium dodecyl sulfate
TCA	Trichloro acetic acid
TEMED	N,N,N',N',-tetramethylethylenediamine
TLC	Thin layer chromatography
TPCK	L-1-tosylamido-2-phenylethyl chloromethyl ketone
Tris	Tris-(hydroxymethyl)-aminomethane
Z	Benzyloxycarbonyl
Z-Arg-Arg-MCA	N-α-benzyloxycarbonyl-L-arginyl-L-arginine-7-amido-4-methylcoumarin
Z-Phe-Arg-MCA	N-α-benzyloxycarbonyl-phenylalanyl-L-arginine-7-amido-4-methylcoumarin

INTRODUCTION

The field of lysosomal proteolysis is currently a subject of intense research in most countries. Considerable progress in the isolation and characterization of these lysosomal proteolytic enzymes during the past few decades have emphasized their wide range participation in vital physiological processes including peptide biosynthesis and protein turnover (Yamamoto & Takahashi, 1993; Sohar & Gyorgy, 1992; Tanaka **et al.**, 1984; Kirschke **et al.**, 1983; Barrett & Kirschke, 1981). The wide distribution of lysosomal cathepsins, a group of proteolytic enzymes, throughout most tissues suggest that they play an important role in protein breakdown by the lysosomal pathway, and their cumulative activities account for more than 50% of total cellular protein degradation (Bohley & Seglen, 1992).

Cathepsin, derived from the Greek word "KATHEPSIN" meaning "to digest," was first proposed by Willstatter and Bamann (1929) for the acid proteinase activity found in the aqueous extracts of various mammalian tissues. Later, they were visualized as a heterogeneous group of enzymes (Bergman & Fruton, 1937; Bergman **et al.**, 1935). Fruton and Bergman (1939) identified three types of cathepsins namely cathepsin I, II and III depending on their activity against low molecular weight substrates Z-Glu-Tyr, Bz-Arg-NH_2 and Leu-NH_2, respectively. These were later renamed cathepsins A, B and C by Tallen **et al.** (1952), as their abilities to degrade synthetic substrates of pepsin, trypsin and chymotrypsin, respectively differed. Another enzyme active against Z-Gly-Phe turned out to be a thiol activated carboxypeptidase and was named as cathepsin IV (Greenbaum & Sherman, 1962).

Mammalian tissues are known to contain several proteinases with similar and somewhat overlapping enzymic properties (Barrett **et al.**, 1981; Barrett, 1977). The availability of specific substrates and inhibitors has helped in identification of more than a dozen cathepsins and related proteinases. Proteinases of lysosomal origin have been shown to belong to one of the four families, namely cysteine, aspartic, serine or metalloproteinases. The classification is based on the nature of the most

prominent functional group in the active site of the enzyme (Yamamoto & Takahashi, 1993).

Cysteine proteinases are characterized by the presence of an essential cysteine residue in their active site (Barrett **et al.**, 1973). They include cathepsins B, H, L and S. The amino acid sequences of rat liver cathepsin B and H (Takio **et al.**, 1983), human liver cathepsin B (Ritonja **et al.**, 1985) and bovine spleen cathepsin B (Meloun **et al.**, 1988) have been worked out. All of them bear close homology among themselves. The three dimensional structure of cathepsin has been shown to have similarity with papain (Hasnain **et al.**, 1992), hence they are grouped under the "Papain Superfamily" (Mordier **et al.**, 1993; Dolenc **et al.**, 1992; Baudys **et al.**, 1991; Takahashi **et al.**, 1984a; Kirschke **et al.**, 1983; Takio **et al.**, 1983).

Cathepsin B exists both in single as well as in double chain forms (Takahashi **et al.**, 1984a). Porcine spleen cathepsin B was found to contain two novel carbohydrate structures which were different from those found on other lysosomal hydrolases (Takahashi **et al.**, 1984a; Takahashi **et al.**, 1984b). The amino acid sequences near the glycosylation site though homologous were found to be unidentical. Thus structural differences suggested the presence of two distinct cathepsin B isozymes (Takahashi **et al.**, 1986a).

Several other thiol proteinases have been identified. Owing to their low content and overlapping physical and enzymic properties their characterization is still a subject of extensive research (Kirschke **et al.**, 1989; Maciewicz & Etherington **et al.**, 1988), these include cathepsin I, J, K, M, N, P and T (Baudys **et al.**, 1991; Takio **et al.**, 1983; Bajkowski & Frankfater, 1983; Evans & Shaw 1983). The fact that overlapping activity is relatively difficult to fully eliminate from a purified cathepsin B preparation, might have been the source of endopeptidase activity in most of the enzyme preparation of purified cathepsin B (Takahashi **et al.**, 1986b). An example of this diverse specificity of cathepsin B is provided by degradation of oxidized insulin B-chain by endopeptidase activity of the

enzyme from human liver (McKay **et al.**, 1983) and total failure of the porcine spleen enzyme to show any activity against this substrate (Takahashi **et al.**, 1986b).

One of the most thoroughly studied thiol proteinase, cathepsin B, exhibits endopeptidase activity with a specificity towards an arginyl-arginyl-X bond, such as in the synthetic substrate of benzyloxy carbonyl arginylarginine-ß-naphthylamide (Barrett **et al.**, 1981; McDonald **et al.**, 1975). It exhibits a trypsin like preference for dibasic sequences in synthetic substrates (Barrett **et al.**, 1981). Cathepsin B also possesses activity of a carboxypeptidase which releases dipeptides sequentially from the carboxyl termini of polypeptides (Baudys **et al.**, 1991; Takio **et al.**, 1983; Bond & Barrett, 1979; Nakai **et al.**, 1978). Because of these specific proteolytic activities, cathepsin B is postulated to be involved in the in vivo processing of protein precursors such as proinsulin and proalbumin (Docherty **et al.**, 1982; Quinn & Judah, 1978; Ansorge **et al.**, 1977).

Tissue Distribution :

Cathepsin B (EC 3.4.22.1) is a ubiquitous lysosomal proteinase present in various tissues as demonstrated by its immunohistochemistry (Tomomasa **et al.**, 1994; Watanabe **et al.**, 1989; Howie **et al.**, 1985). The endogenous levels of cathepsin B are found to vary in different animal tissues (Bando **et al.**, 1986; Kominami **et al.**, 1985) in the order of kidney>spleen>liver>vagina>adrenal glands (Kominami **et al.**, 1985). Heart, skeletal muscle and testes have been reported to contain low levels of cathepsin B (Kominami **et al.**, 1985). In the soluble extracts of peripheral blood cells, the macrophages contain the higest amount of cathepsin B (Kominami **et al.**, 1985).

Gene Structure :

Cathepsin B is encoded by a single copy of gene under the control of a "house keeping" type promoter. The primary sequence of rat

cathepsin B consists of 330-340 amino acid residues and contains a typical signal sequence (17-21 residues), pro-peptide sequence (62-96 residues) and mature enzyme region (220-245 residues). Pulse chase experiments showed that the earliest form of cathepsin B is synthesized as a 39 kDa precursor polypeptide that is processed first to a single chain form of 29 kDa within 1 hour and then to a double chain form after a period of 21 hours.

The mouse cathepsin B gene consists of 10 exons and 9 introns spanning about 20 Kb in length. The 5' upstream region and exon 1 are GC rich. TATA and CAAT motifs adjacent to the transcription start site are not evident. These properties are characteristic of mammalian "housekeeping genes" (Gong **et al.**, 1993; Qian **et al.**, 1990). The 5' untranslated region (leader) is interrupted by a large intron. The second exon (exon-2) contains the translation initiation site. Nucleotide sequence analysis revealed three different leader regions. Murine cathepsin B has been shown to possess multiple promoters which may lead to expression of multiple mRNAs, differing in their leader sequences (Rhaissi **et al.**, 1993). Multiple mRNA species produced by alternative splicing in the 5' and 3' untranslated regions suggest that the expression of cathepsin B may be regulated, in part at least, at the level of mRNA processing (Gong **et al.**, 1993).

The expression, post-translational processing, and targeting of cathepsin B is frequently altered in transformed and malignant cells (Sloane, 1990). Cathepsin B lacking exon 2 are predominant in human tumours. In addition, human breast and colon carcinomas and a human melanoma contain a cathepsin B transcript that is also missing in exon 3, encoding the signal peptide and 7 amino acid residues of the activation propeptide. An in vitro transcription/translation assay demonstrated that this message could be translated from an internal methionine codon (residue 52) producing a 32 kDa product lacking the signal peptide and more than half the propeptide. It also indicated that the variant messages differ in their rate of translation (Gong **et al.**, 1993). Alternative splicing of cathepsin B pre-mRNA may account for the diversity of form and location of cathepsin B in human tumours (Gong **et al.**, 1993).

Biosynthesis and Proteolytic Processing:

Cathepsin B is a glycoprotein and is synthesized in the lumen of the rough endoplasmic reticulum prior to its glycosylation with mannose linked phosphate residue as the N-linked oligosaccharide. In many cells, the targeting of soluble lysosomal enzymes depends on synthesis of mannose-6-phosphate residues in their oligosaccharide side chains. These residues are recognized by specific receptors that deliver the enzymes from the trans-golgi apparatus to pre-lysosomal compartments (Kornfeld, 1992; von Figura **et al.**, 1986). Signals for the phosphorylation of the lysosomal enzymes consist of a surface patch of several amino acid residues. Among these, lysine appears to play the most prominent role (Baranski **et al.**, 1991 and 1990). In cathepsin B the presence of carbohydrate moieties are found mostly in the heavy chain, linked with Asn-113 side chain (Musil **et al.** 1991; Ritonja **et al.**, 1985; Takio **et al.**, 1983).

Like other lysosomal cathepsins, cathepsin B too is synthesized as a latent precursor form. Proteolytic maturation of the latent pro-enzyme to the active enzyme may occur in endosomes between trans Golgi network and lysosomes or in lysosomes after transport (Fox **et al.**, 1992). Moreover, procathepsin B is also known to be autoactivated under an acidic milieu (Mach **et al.**, 1994), although cathepsin D has also been reported to act as a converting enzyme for the proteinase (Nishimura **et al.**, 1990). The available information on nucleotide sequences of rat, human, bovine and porcine cathepsin B suggest that the enzyme is synthesized as a larger molecular mass precursor (Ferrara **et al.**, 1990; Fong **et al.**, 1986; Chan **et al.**, 1986). Cathepsin B from human, rat and mouse is synthesized as a 339 amino acid polypeptide chain, which is processed to the mature single chain molecule of 254 amino acid residues (Kominami **et al.**, 1988; Nishimura & Kato, 1987). In mammalian tissues, most of the active form of cathepsin B is found as a two-chain molecule consisting of 47 (or 49) and 205 (or 204) residue polypeptide chains (light and heavy chain) covalently cross-linked by a disulfide bridge (Musil **et al.**, 1991; Kominami **et al.**, 1988). Cathepsin B from porcine spleen is predominantly a two chain protein (Takahashi, 1984 a) in contrast to rat liver enzyme which contain equal amounts of single and two chain species (Takio **et al.**, 1983).

Cathepsin B has also been suggested to exist in isozyme forms with isoelectric pHs and molecular weights in the range of 4.5-5.5 and 24-29 kDa, respectively (Barrett and Kirschkie, 1981). Takahashi **et al.**, 1986a, reported two isozyme forms of cathepsin B from porcine spleen which differed in their carbohydrate structure. Differences in the kinetic properties of the enzyme from synovial fluid and bone marrow cells have also been attributed to the possible existence of isozymes in these tissues (Van Noorden **et al.**, 1989).

Although the physiological significance for occurrence of isozymes in cathepsin B is not very clear since the biological functions of these are not known, it is well established that isozyme composition patterns change depending on the degree of differentiation of tissues. A number of such changes have also been reported for liver enzymes (Weinhouse **et al.**, 1976).

Specificity, Kinetics and Catalytic Mechanism:

Cathepsin B from different sources is stable within the pH range of 4.5-7.5 (Jiang **et al.**, 1994; Baricos **et al.**, 1988). It does not appear to be stable in vitro at pH values above 6.5 and is irreversibly denatured in the alkaline pH zone (Bromme **et al.**, 1993; Khan **et al.**, 1986). Instability seems to be mainly due to rapid autolysis of the enzyme at pH values above 6.5 (Buck **et al.**, 1992).

Cathepsin B has been shown to be involved in the maturation of secretory proteins in Golgi vesicles and secretory vacuoles, in addition to its role in secondary lysosomes (Taugner **et al.**, 1985; Steiner **et al.**, 1983). Although it is generally anticipated that extra cellular proteolysis is prevented by the instability of lysosomal cysteine proteinases at neutral pH, some investigations have shown that cathepsin B has a potent, but short lived, destructive potential at pH levels up to 8.0 (Hasnain **et al.**, 1992; Maciewicz **et al.**, 1990; Sheahan **et al.**, 1989; Keppler **et al.**, 1988;Mort **et al**,. 1984). The activity of cathepsin B at alkaline pH in malignant tumours or lactating mammary glands may be due to the fact that, cathepsin B is unlikely to be inactivated when associated with

membranes (Erdel **et al.**, 1990) or protein substrates (Sloane, 1990; Lah **et al.**, 1989).

Cathepsin B purified from different sources exhibit endo- and exo-peptidase activities towards natural and synthetic substrates (Takahashi **et al.**, 1986a, b). It is possible that the domain of Gly-Gly-His-Ala contribute to the unique specificity of cathepsin B as a peptidyl dipeptidase in contrast to cathepsin H having domain of Val-Asn-His-Ala, which acts as an amino peptidase (Willenbrock & Brockleharst, 1985).

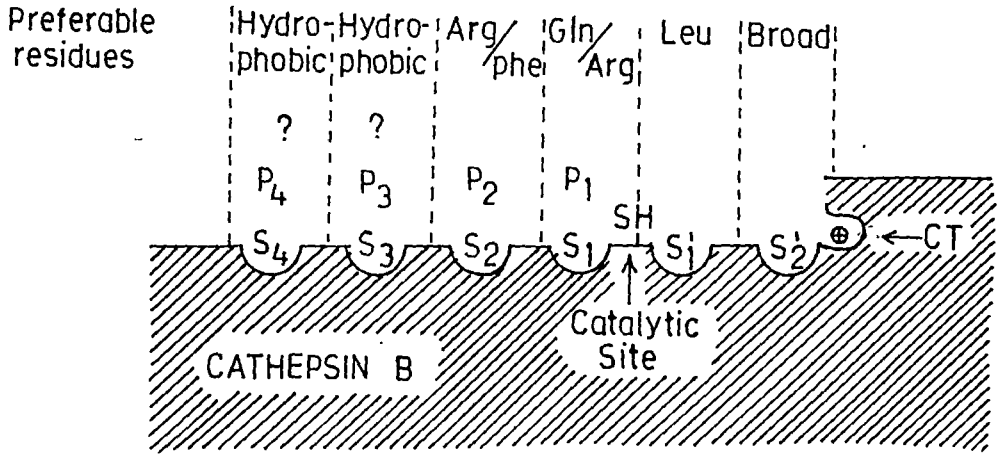
Most of the synthetic substrates for cathepsin B have Arg preceding the peptide bond to be cleaved, designated as P_1 (nomenclature as per Schecter & Berger, 1968) (Fig. 1) as in the case of trypsin (Barrett & Kirschke, 1981). However, binding of a P_2 (i.e. P_1 + 1) residue generally increases the K_{cat}/K_m value and thus P_2 is perhaps the most important residue in the substrate, as in case of papain (Barrett & Kirschke, 1981). Usually the N-blocked Arg- derivatives such as 2-napthylamine, 4-nitroaniline, amide ethyl ester and 4-nitrophenyl ester, have been used for cathepsin B assay (Ahmad **et al.**, 1989; Khan **et al.**, 1986; Fazili & Qasim, 1986; Takahashi **et al.**, 1984a; Barrett & Kirschke, 1981; Singh & Kalnitzky, 1978). These substrates are however good for other similar lysosomal cysteine proteinases too, such as cathepsin H (Takahashi **et al.**, 1986b,1984b) and cathepsin S (Takahashi **et al.**, 1986b). Similar molecules with a pair of Arg residues are good substrates for cathepsin B (Knight, 1980; Davidson & Poole, 1975; McDonald & Ellis, 1975). Cathepsin B is also capable of degrading protein substrates like soluble and insoluble collagen (Burleigh **et al.**, 1974), oxidized B-chain of insulin (Ansorge **et al.**, 1977), denatured hemoglobin (Agarwal & Khan, 1987b; Fazili & Qasim, 1986), casein and BSA (Ahmad & Khan 1990; Agarwal & Khan, 1987b) and fibronectin (Guinec **et al.**, 1993) under in vitro conditions.

Model of Active Site :

Cathepsin B is roughly disc shaped with a diameter of 50 Å and thickness of 30 Å. It has two domains namely L and R. Residues 12-134, 153-158 and 250-252 of the enzyme polypeptide chain form the L

Fig.1 Schematic representation of the acive site of cathepsin B (Takahashi **et al.**, 1986b).

Sulfhydryl group at the catalytic site is indicated by SH. Substrate side chain(P_1-P_n) binding sites to the left of SH are designated as S_1-S_4 and to the right as S_1' and S_2'. The COOH- terminal carboxyls in the substrates are supposed to bind to the positive charge, (+), in the site designated as CT. The preferable amino acid residues for each side-chain binding pockets are also depicted.


Preferable residues
Hydro-phobic
Hydro-phobic
Arg/phe
Gln/Arg
Leu
Broad
?
?
P4
P3
P2
P1
SH
S4
S3
S2
S1
S'1
S'2
CT
Catalytic Site
CATHEPSIN B

domain while rest of the residues form the R domain. A cleft present between these two domains with Cys 29 residue located inside, forms the active site (Sumiya **et al.**, 1992; Musil **et al.**, 1991).

Cysteine proteinases such as cathepsin B, H, L and S can be distinguished from each other depending on their ability to accommodate different amino acid side chains. In these proteinases, substrate specificity appears to reside at the P_2 rather than the P_1 position (Fig. 1). Cathepsin B is interesting in this regard since it accepts an arginine residue in the P_2 position whereas most other members of the cysteine proteinase family do not (Hasnain **et al.**, 1992).

The mechanism of cathepsin B specificity and catalysis is still not fully understood. However, the role of certain charged residues in substrate binding have been postulated based on alignment of cathepsin B sequences with papain in particular. In cathepsin B, a large number of charged residues which could have an influence on enzyme activity, are present in the vicinity of the active site cleft. Cys 29 and His 199 are two active site residues absolutely required in their deprotonated and protonated forms, respectively for catalysis. A glycine residue is present at position 198 in cathepsin B for all species where sequence information is available. Greater flexibility of glycine relative to aspartic acid in papain leads to a broader S_2 pocket in cathepsin B (Hasnain **et al.**, 1992) (Fig. 1). It has been suggested that His 110 and His 111 may play a crucial role in exopeptidase activity of cathepsin B since the protonated imidazolium groups could interact with the negatively charged substrate C-terminal carboxylate (Hasnain **et al.**, 1993; Sumiya **et al.**, 1992; Baudys **et al.**, 1991; Musil **et al.**, 1991).

The number of disulfide bridges have been reported to be 7 and 6 in bovine and human cathepsin B, respectively (Musil **et al.**, 1991; Baudys **et al.**, 1990; Ritonja **et al.**, 1985). Two cysteine residues of cathepsin B namely Cys 29 and Cys 240 are unpaired. Cys 29 is topologically equivalent to the reactive cysteine in all cysteine proteinases, whereas Cys 240 is located close to the R-domain and is unique in cathepsin B (Musil **et al.**, 1991).

Proteinase Inhibitors :

By judicious choice of amino acids to occupy the P_1 to P_n position of the inhibitors (nomenclature of Schecter and Berger, 1968) (Fig. 1) so as to fulfill the primary and subsite specificity requirements of individual members of serine and cysteine proteinases, it has been possible to obtain reagents that exhibit pronounced selectivity of action (Walker, **et al.**, 1993). Peptide sequences in which C-terminal amide (-CONH-) or acid (-COOH) functional groups have been replaced by electrophilic moieties such as aldehyde (-CHO) (Thompson, 1973; Westerik & Wolfenden, 1972), trifluoromethyl ketone ($-COCF_3$) (Stein **et al.**, 1987; Imperiali & Abeles,1986) and α-oxoester (-COCOOR) (Peet **et al.**, 1990; Medhi **et al**, 1990), have yielded potent and reversible inhibitors of serine and cysteine proteinases.

Peptidyl aldehyde inhibitors such as leupeptin, chymostatin, antipain, calpeptin etc. bind covalently but reversibly to the active site nucleophile of cysteine proteinases as hemithioacetals (Medhi 1990; Giordano **et al.**, 1990). Recently, it has been demonstrated that homogenates of cells and tissues harvested after culture in leupeptin showed an increase in activity of cathepsin B. This may be attributed to the fact that lysosomal cysteine proteinases are actually involved in their own degradation. However, leupeptin decreases the degradation of the lysosomal enzymes including cathepsin B by its inhibitory activity towards lysosomal cysteine proteinases (Montenez **et al.**, 1994).

There are several irreversible inhibitors specific to cysteine proteinases which include thiol blocking reagents such as iodoacetic acid, iodoacetamide, p-chloromercuric benzoic acid, peptidyl fluoromethyl ketones, diazomethyl ketones, epoxysuccinyl peptides like E-64 and their derivatives (Gour-Salin **et al.**, 1993). Several analogues of E-64 which are potential candidates for therapeutic applications have been synthesized, of which a synthetic analogue L-trans-epoxysuccinyl-leucinamido (3-methyl) butan 4, named E-64c or Ep-475 (Barrett **et al.**, 1982; Tamai **et al.**, 1981; Hashida **et al.**, 1980), containing a neutral isopentyl group in P_3 proved to be substantially more efficient than E-64 as an inhibitor of cathepsin B and

L (Giordino **et al.**, 1993). The irreversible inactivation of the enzyme was shown to proceed with the formation of a covalent bond between the nucleophilic thiolate of the catalytic cysteine and C-3 of the epoxysuccinyl unit (Giordino **et al.**, 1993).

Physiological inhibitors particularly cystatins are known to play a major role in regulation of cysteine proteinases. Cystatins which belong to cysteine proteinase inhibitors (also called thiol proteinase inhibitors) are classified into three groups namely, cystatin I (or stefins), cystatin II (or cystatins) and cystatin III (or kininogens). This classification is based on molecular size, number of disulfide bonds, presence of carbohydrate moieties, primary structure and subcellular localization (Barrett **et al.**, 1986).

Cystatin C, a member of cystatin family II, is expressed in a variety of tissues and display a high affinity for cathepsin B (Corticchiato **et al.**, 1992; Barka **et al.**, 1992). It therefore appears to be a potentially important physiological inhibitor of cysteine proteinase activity in extracellular fluids (Abrahamson **et al.**, 1991, 1986).

Pathological and Physiological Significance :

Lysosomal cysteine proteinases have been implicated in intracellular protein degradation and are believed to be involved in a variety of physiological and pathological processes (Katunuma **et al.**, 1983; Sher **et al.**, 1981; Stracher **et al.**, 1978; Huisman **et al.**, 1974). Cathepsin B is involved in platelet aggregation (Sloane **et al.**, 1992) and post translational processing of proteins (Sohar & Katona, 1992; Mizuno **et at.**, 1982). It also takes part in prohormone activation. In fact, cathepsin B and H have been demonstrated to be localized in secretory granules of some peptide hormone producing cells including those producing active renin (Sano **et al.**, 1993; Uchiyama **et al** ., 1991, 1989). It is also involved in parathyroid hormone (PTH) proteolysis. By in vitro studies, cathepsin B has been demonstrated to cleave PTH into various forms of PTH fragments (MacGregor **et al.**, 1986; Anderson **et al.**, 1986). Storage granules in rat parathyroid cells have been found to be fused with small vesicles containing cathepsin B and H, which possibly participates in the regulation of PTH

level by degrading newly synthesized PTH in the granules (Uchiyama **et al.**, 1994). Cathepsin B is reported to play an important role in neuronal degeneration or the dysfunction of brain regions in relation to aging (Banay-Schwartz **et al.**, 1992; Kenessey **et al.**, 1989).

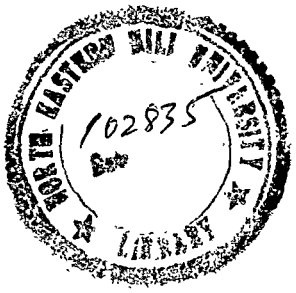
Pathophysiological changes and cellular transformation have been shown to be accompanied by a redistribution of cathepsin B to secretory vesicles (Achkar **et al.**, 1990), the nucleus (Pietras & Roberts, 1981) and the plasma membrane (Rozhin **et al.**, 1989). Secretion of several different forms of cathepsin B including higher molecular weight latent and active forms (Maciewicz **et al.**, 1989; Docherty & Philips, 1988; Mort **et al.**, 1983, 1980) which is attributed to post-translational processing leading to changes in amount and composition of oligosaccharide side chains (Spiess **et al.**, 1994; Nishimura **et al.**, 1988) and extent of proteolytic modifications (Hara **et al.**, 1988; Chan **et al.**, 1986), are some of the characteristics of transformed cells. In some human tumours a part of this diversity and location of cathepsin B may be due to alternative splicing of cathepsin B pre-mRNA (Gong **et al.**, 1993).

Cysteine proteinases like cathepsin B and L can activate latent proteinase activity associated with fibronectin in basement membranes leading to a metastatic cascade which may be an important factor in the in vivo basement membrane dissolution observed in tumour invasion (Guinec **et al.**, 1993). Many tumour cells are glycolytic and secrete large amounts of lactic acid into the extracellular medium providing a micro-acidic environment for optimal activity of lysosomal proteinases as they are most active at acidic pH (Young & Spevacek, 1993). Cathepsin B has also been implicated in other pathological conditions such as proteinuria and renal disorders (Baricos **et al.**, 1990), myocardial infarction (Laszlo **et al.**, 1993; Bolli **et al.**, 1983) and pulmonary emphesema (Trabandt **et al.**, 1991).

In spite of extensive research, the mechanism of action of cathepsin B and its catalytic specificity are still not well understood. Thus to understand the role of this proteinase in a variety of biological processes,

detailed information on the physicochemical and catalytic properties is required. The possibility of the existence of cathepsin B isozymes and their roles also require a systematic investigation. In view of this, we have undertaken the present work to isolate and study the properties of cathepsin B from a hitherto unstudied source i.e. goat spleen.

EXPERIMENTAL



A. MATERIALS :

Proteins used :

Cathepsin B was isolated from goat spleens collected from local abattoirs. Bovine serum albumin, ovalbumin, α-chymotrypsinogen, myoglobin, ribonuclease A, cytochrome C, carboxypeptidase A, and rabbit muscle aldolase were purchased from Sigma Chemical Company, U.S.A. Bovine milk casein was obtained from Sisco Res. Lab.,India.

Reagents used in end group analysis :

Dansyl chloride, iodoacetamide and standard amino acid kit were purchased from Sigma Chemical Company, U.S.A. Micro polyamide sheets (5.0x5.0 cm) were the products of Pierce Chemical Company, U.S.A. Organic solvents viz., benzene, amyl-alcohol, N-butanol, tertiary butanol, pyridine, toluene, acetone, ethanol, methanol, chloroform, glacial acetic acid, formic acid etc. were purchased from BDH, India.

Chromatography media :

Sephadex (G-25,G-75 and G-100), Blue Dextran 2000, DEAE Cellulose 52, CM-Sephadex C-50 and ConA-Sepharose 4B were obtained from Pharmacia Biotek, Uppsala, Sweden. Amberlite MB-3 was obtained from Sigma Chemical Company, U.S.A.

Reagents used for polyacrylamide gel electrophoresis :

Acrylamide, N-N' methylene bisacrylamide, N,N,N',N'-tetramethylethylene diamine, 2- mercaptoethanol, Coomassie Brilliant Blue R-250, glycine, Tris (Hydroxymethyl aminomethane), sodium dodecyl sulfate purchased from Sigma Chemical Company, U.S.A. were used for polyacrylamide gel electrophoresis in the presence and absence of SDS. Ammonium persulfate, sucrose and glycerol were obtained from E. Merck,

India and amidoschwarz and bromophenol blue were the products of BDH, England.

Other reagents :

α-methyl-D-glucopyranoside, succinic anhydride, maleic anhydride, urea, guanidine hydrochloride, N-acetyl-L-cysteine, BANA, BAPNA, Leu-BNA, Arg-BNA, 2-naphthylamine, p-nitroaniline, DTNB, FCA, IFA, antipain, E-64, leupeptin, pepstatin A, cysteine base, cysteamine-HCl, DTT, 2-mercaptoethanol, glutathione, thioiglycerol, iodoacetic acid, mercuric chloride, manganese chloride, sodium azide and calibration mixture for amino acid analysis were obtained from Sigma Chemical Comapny, U.S.A.. Coomassie Brilliant Blue G-250, Brij 35, o-pthalaldehyde were the products of Serva Biochemicals, U.S.A. Z-Arg-MCA, Z-Arg-Arg-MCA, Z-Phe-Arg-MCA amd MCA were the products of Peptide Institute, Inc., Japan. TCA, chromic acid, N-1-naphthylethylene diamine-HCl and standard buffer tablets of different pHs were purchased from BDH, England. Agarose, sodium nitrite, ammonium sulfamate, EDTA, histidine, papain, methyl cellusolve, ninhydrin were from Sisco Res. Lab., India. D-glucose, ascorbic acid, potassium ferricyanide and orthophosphoric acid were pruchased from Qualigens, Bombay, India. Phenol, perchloric acid, sodium hypochlorite, potassium sulfate and ethanol were the products of Fluka Chemical AG, Switzerland. All other reagents were of analytical grade or the best commercially available.

Miscellaneous :

Dialyser tubings of diffferent diameters were purchased from Sigma Chemical Company, U.S.A. Millipore filters (pore size 0.22 μM, 0.45 μM), filter papers of different diameters and pH papers were obtained from Whatman Co., England. Nitrogen gas was supplied by I.O.L India. Rabbits were purchased from I.C.A.R farm, Shillong, India.

B. METHODS :

1. pH measurements:

All pH measurements were made at room temperature (15-27°C) using a Control Dynamics digital pH meter, model APX 175 E/C. Routine calibration of the pH meter was done using standard buffer tablets (BDH) of different pHs.

2. Optical measurements :

a) Absorption measurements :

Light absorption measurements in the ultraviolet region as well as the visible region were made on Jasco UVIDEC-610 double beam, spectrophotometer using quartz (in UV region) or glass (in visible region) cuvettes of 1 cm path length. All measurements were done at room temperature, unless stated otherwise.

b) Fluorescence measurements :

Fluorometric studies were done in a Shimadzu RF 540 spectrofluorophotometer fitted with a thermo-regulated sample chamber and automatic polarizors. Fused quartz cuvettes of 1 cm path lengths were used for all the experiments.

3. Centrifugation:

Centrifugation was carried out at 4°C either in a Beckman refrigerated centrifuge Model J2-21 or in a Remi cooling centrifuge Model C-24. Microcentrifugation was done in an Eppendorf microcentrifuge.

4. Determination of protein concentration:

Protein estimations were done either by the dye binding method of Bradford (1976), using BSA as standard, or directly by measuring their absorbance at 280 nm using values of their specific extinction coefficients.

a) Dye-binding method :

The method originally developed by Bradford (1976) and modified by Bio-Rad Ltd., U.S.A (1979), consists of the following steps :

i) Preparation of colour reagent : 100 mg of Coomassie Brilliant Blue G-250 was dissolved completely in 50 ml of ethanol (95%). To it 100 ml of 85% (w/v) orthophosphoric acid was added. The contents were thoroughly mixed and transferred to a dark bottle with a tight stopper. This was the Bradford stock reagent which was kept refigerated for long term use.

The working solution was prepared by diluting 15 ml of the stock reagent described above to 100 ml with distilled water. This reagent was filtered through Whatman filter paper #1 and kept for use at room temperature, with a bench life of approximately two weeks.

ii) Assay of proteins : To 1.0 ml of protein solution 5.0 ml of Bradford working solution was added and mixed thoroughly. Colour was allowed to develop for 10-15 min at room temperature and the intensity of the colour was determined at 590 nm within 30 min against a reagent blank prepared from 1.0 ml of the appropriate buffer and 5.0 ml of the reagent. Protein concentration was determined with the help of a standard curve prepared as stated above with varying concentrations of BSA.

b) Spectrophotometric method :

The optical density of different protein solutions measured at 280 nm was divided by their respective specific extinction coefficient ($E^{1\%}_{1cm}$) at 280 nm. The value of the quotient gave the amount of protein in grams per 100 ml of the solution. Correction for possible light scattering were routinely made by measuring the absorbance of the protein solution in the wavelength range, 360-340 nm and extrapolating those values into the absorbing region.

5. Chromatography:

a) Gel filtration :

Gels were prepared by swelling dry powder of Sephadex in excess of distilled water for a period recommended by the manufacturers. The fines of

the gel slurry were removed by decantation after which it was degassed to remove the trapped air. Glass columns previously washed with detergent, chromic acid and water, were mounted vertically. Well mixed gel suspension was then carefully poured into the column with the help of a clean glass rod. The gel was allowed to settle under gravity for about an hour and then constant hydrostatic pressure was applied through a peristaltic pump to settle the gels at a flow rate of 15 cm/hr. The column was finally equilibrated by passing excess (2-3 times the total bed volume of the column) of equilibrating buffer at a constant flow rate of 25 ml/hr with the help of a peristaltic pump. Homogeneity of the packed gel bed was checked by monitoring the progress of a narrow band of 0.2% (w/v) Blue Dextran 2000 and $K_3Fe(CN)_6$ solution (2 mg/ml). The elution of the former gave the void volume (V_o), while that of the latter gave the total volume (V_t) of the packed column. Before application of the sample, the buffer solution from the top was removed by suction leaving only about 2 mm of the buffer above the gel. 2-5 ml of the sample containing 5-10 mg of the protein was then carefully applied on the column with the help of a thin tubing and allowed to pass down the column, taking care not to allow the gel surface to get dry. The upper surface of the column was rinsed with 4-5 ml of eluting buffer and allowed to run with a constant flow rate of 20-25 ml/hr, after connecting the column to a buffer reservoir. Fractions (3-5 ml) were collected and monitored for their protein concentration and enzyme activity (if applicable) with suitable methods.

b) Thin layer chromatography:

Thin layer chromatography was performed on micropolyamide sheets (Pierce Chem Co.) of 5 x 5 cm. Samples were spotted repeatedly about 5 mm above the bottom line with the help of finely drawn tips of capillary tubes. Care was taken so that the spots did not exceed 2 mm in diameter. Ascending chromatography was then carried out in 150 ml chromatographic chambers containing the appropriate solvent systems. After the completion of the run the sheets were removed and dried at room temperature. The chromatograms were detected as fluorescent spots by placing the plates under

ultraviolet lamp and the R_f values were computed by dividing the distance moved by the samples with that of the solvent front.

6. Gel electrophoresis:

Electrophoresis on polyacrylamide gels were done in the absence and presence of SDS according to the procedures described by Davis (1964) and modified technique of Laemmli (1970) respectively.

a) Polyacrylamide gel electrophoresis (PAGE):

Clean gel tubes (0.5x1.0 cm) were mounted vertically in a stand after sealing the lower ends. A gel solution was prepared by mixing 2 volumes of solution A (containing 14.4 % acrylamide and 0.6 % N,N',bisacrylamide), 1 volume of solution B (1.5 M Tris-HCl buffer, pH 8.9, containing 110 µl of TEMED) and 1 volume of freshly prepared ammonium persulfate solution (2.7 mg / ml). The resulting solution gave gels with a cross linking of 7.5 % . 50-100 µg of protein (20-80 µl) containing 40 % glycerol (or sucrose) and a small amount of bromophenol blue was carefully applied on the top layer of the gel. The electrophoresis was carried out with an anodic current of 2 mA/tube for 2-3 hrs, using Tris-glycine buffer, pH, 8.3 (prepared by dissolving 2.88 grams glycine and 0.6 gms Tris in 1 litre distilled water). The gels were stained with 0.01% amido black (or Coomassie Brilliant Blue R-250) for about 40 min and finally destained by a destaining solution containing 7.0 % acetic acid and 5 % methanol at 37°C repeatedly until the background was clear.

b) Sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE):

Polyacrylamide gel-electrophoresis of the proteins in presence of 1.5 % sodium dodecyl sulfate was done both in presence and absence of 0.1 % 2-mercaptoethanol. A 12.5 % gel was prepared by mixing 10.5 ml water, 8.25 ml solution A (30 % Acrylamide, 0.8 % N, N'-bisacrylamide.), 1.25 ml solution B (18.7 gms Tris + 2.0 ml 20 % (w/v) SDS in 100 ml water carefully adjusted to a pH of 8.8 with 6.0 N HCl. 0.38 ml freshly prepared

ammonium persulfate solution (20 mg/ml) and 0.03 ml TEMED. Protein samples were denatured in 1.0 % SDS by heating in a boiling water bath for 10 min followed by overnight incubation with 0.1 % 2-mercaptoethanol (if required). Electrophoresis of the denatured protein samples were performed either at pH 6.8 using 60 mM sodium phosphate buffer or at pH 8.3 using 60 mM Tris-HCl buffer, containing 1.5 % SDS in the running buffers. Before loading the samples in the gels, a pre-run was done for 30 min at a constant current of 2 mA/gel. The protein samples (50-100 μg) mixed with 20 % glycerol and 0.1 % bromophenol blue was applied on each gel surface. Electrophoresis was performed at a constant current of 3 mA/tube. The gels were stained with Coomassie Brilliant Blue R-250, and finally destained in a solution containing 7 % acetic acid and 5 % methanol. The relative mobilities (R_m) of the electrophoresed protein samples were determined by the standard procedure of Laemmli (1970) using the expression:

$$Rm = \frac{\text{Distance traversed by the protein band (cm)}}{\text{Distance traversed by the dye band (cm)}}$$

7. Isolation and purification of cathepsin B:

The isolation and purification of cathepsin B from goat spleen was performed according to the method of Takahashi **et al.**, (1983) and Ahmad **et al.**, (1989), by incorporating suitable modifications. The procedure consisted of the following steps:

a) Homogenization:

Goat spleens from freshly slaughtered goats were collected in ice from local abattoirs, and kept frozen at -20°C before use. The frozen spleens were thawed and washed with excess amount of distilled water followed by 1mM EDTA solution. The soft mass of the tissue weighing about 350 gms (obtained from 500 gms of spleen after removing fats, peripheral membranes and connective tissues) was homogenized with 150 ml of 3% NaCl solution containing 1mM EDTA and 15 mM HCl (pH 1.8). This was kept suspended for 6 hrs at 4°C with continuous stirring.

b) Acid extraction :

The pH of the homogenate obtained above was adjusted to 3.8 by gradual addition of chilled 2N HCl and left for continuous stirring at 4°C for another 8 hrs. The content was then centrifuged at 14,000 rpm for 20 min and the clear supernatent was collected.

c) Ammonium sulfate fractionation :

The clear supernatent obtained after acid extraction was subjected to salt fractionation. The protein fraction precipitating between 40-75% ammonium sulfate saturation was collected and dissolved in minimum amount of chilled distilled water. This was then extensively dialysed against chilled distilled water followed by 50 mM sodium acetate buffer, pH 5.0, containg 1 mM EDTA and 0.02% sodium azide. The protein solution after dialysis was again centrifuged at 14000 rpm for 20 min and the clear supernatant thus obatined was used for further purification.

d) Gel chromatography on Sephadex G-75:

The protein solution obtained through salt fractionation was concentrated and chromatographed on G-75 column (2.6x90 cm) pre-equilibrated with 50 mM sodium acetate buffer, pH 5.0, containing 1 mM EDTA and 0.02 % sodium azide. The fractions were monitored spectrophotometrically for protein concentration at 280 nm and for enzyme activity using BANA as the substrate. The enzymatic active fractions were pooled and concentrated.

e) Ion-exchange chromatography on DEAE-Cellulose column:

The enzymatically active fractions collected after gel filtration were dialysed against 20 mM Tris-acetate buffer, pH 6.0, containing 1 mM EDTA and 0.02% sodium azide, and passed through a DEAE-Cellulose column (1.6x12 cm). After washing the column with the above buffer, the bound fractions of cathepsin B (and similar proteinases) were eluted step wise with the same buffer, containing 0.2, 0.5 and 1.0 M NaCl. The protein fractions eluting at about 0.2 M NaCl, were collected.

f) Ion-exchange chromatography on CM-Sephadex column:

The enzymatically active fractions obtained after DEAE-Cellulose chromatography were thoroughly dialysed against 20 mM sodium acetate buffer, pH 4.8, containing 1 mM EDTA and 1.4 mM 2-mercaptoethanol and applied on to a CM-Sephadex column (1.6x10 cm), equilibrated with the above buffer. The column was washed with the same buffer at pH 4.8 followed by the stepwise elution of bound protein fractions with the above buffer, at pHs 5.6, 6.0 and finally using 1 M NaCl at pH 6.0. Protein estimation and enzyme assay of the eluted fractions were measured. The protein fraction eluting at pH 5.6 were collected.

g) Affinity chromatography on ConA-Sepharose 4B column:

The enzymatically active fractions which eluted at pH 5.6 were dialysed against 0.05 M sodium acetate buffer, pH 6.0, containing 0 2 M NaCl, 1.4 mM 2-mercaptoethanol and 1 mM each of $CaCl_2$ and $MnCl_2$ and was applied on a ConA-Sepharose 4B column (1.5x7cm). A fraction of the enzyme preparation was not retained in the column and eluted with the above mentioned buffer, which was termed as GSCB-I. The bound fraction was eluted from the column with 0.5 M methyl-α-D-glucopyranoside and was termed as GSCB-II. Both the fractions showed cathepsin B activity and were pooled separately.

Each of the two fractions of cathepsin B thus obtained were concentrated and dialysed against 50 mM sodium acetate buffer containing 1 mM EDTA and 0.02% sodium azide, pH 5.0, and was used for further studies.

8. Kinetic studies :

Cathepsin B assays with synthetic substrates:

a) Fluorometric assay of cathepsin B with BANA :

Enzymatic assay of cathepsin B using α-N-benzoyl-DL-arginine-2-naphthalamide (BANA) as the substrate was done according to the method described by Khan **et al.**, (1986). Briefly, to 0.1 ml of enzyme solution

containing appropriate amount of enzyme, 1.9 ml of activator buffer (20 mM sodium phosphate buffer, pH 6.5, containing 2.0 mM EDTA and 2.0 mM 2-mercaptoethanol) was added and incubated at 37°C for 30 min. The reaction was initiated by addition of 1 ml of the substrate solution. (0.1% BANA, dissolved in 3% DMSO and diluted with the activator buffer to the desired concentration). BANA hydrolase activity was measured fluorometrically by monitoring the release of 2-naphthalamine continuously for 30 min at 37°C, using excitation and emission wavelengths of 335 and 410 nm, respectively. The amount of 2-naphthalamine released was calculated using the standard curve prepared with 2-naphthalamine in the same manner, as described by Barrett and Kirschke (1981). One unit of enzyme activity was defined as the amount of enzyme required to release 1 µmol of 2-naphthalamine per min at 37°C.

b) Colorimetric assay of cathepsin B with BANA:

The colorimetric assay of BANA hydrolase activity was performed according to the method of Martineck **et al.**, (1964) with modifications. Briefly, 1.0 ml of enzyme preparation in activator buffer was incubated for 30 min at 37°C. 0.5 ml of the activated enzyme solution was pipetted out in a separate test tube for preparing the control. To the remaining 0.5 ml enzyme solution, 0.5 ml of substrate solution (0.1% BANA in 3% DMSO, prepared as described above) was added, mixed by inversion and incubated at 37°C for 30 min or 1 hr. The reaction was then stopped by the addition of 0.5 ml of 4.0 N HCl and the amount of 2-naphthalamine thus released was determined by diazotization method as described below:

The reaction mixture (1.5 ml), mixed with 0.5 ml sodium nitrite (0.2% w/v) was left for 3 min. Following this, 1.0 ml of ammonium sulfamate solution (0.5% w/v) was added and mixed properly. After another 3 min of standing at room temperature, 2.0 ml of dye (N-1-napthylethylenediamine-dihydrochloride, 0.05% w/v in absolute alcohol) was added to it. The colour was allowed to develop for at least 45 min, and the blue colour thus formed was meassured at 540 nm against a suitable enzyme blank. The blank was

prepared in the same way except that HCl was added to the enzyme before the addition of the substrate.

The amount of 2-napthylamine released was calculated from the standard curve prepared in a similar manner with varying concentrations of 2-napthylamine.

c) Colorimetric assay of cathepsin B with BAPNA:

To 0.5 ml of preactivated enzyme solution 0.5 ml BAPNA solution (0.1% prepared the same way as detailed above for BANA) was added. The reaction was allowed to proceed at 37°C for 30 min or 1 hr and finally terminated by addition of 1.0 ml of 30% acetic acid. The product (4-nitroaniline) released was determined spectrophotometrically at 400 nm using a suitable reagent enzyme blank prepared in the same way, except that the substrate was added after addition of acetic acid to the enzyme solution. The product released was quantitated through a standard curve prepared in the same way with varying concentrations of 4- nitroaniline and was read against a suitable reagent blank.

d) Fluorometric assay of 7-amino-4-methyl-coumarin releasing substrates:

Enzymatic assay against 7-amino-4-methyl-coumarin releasing substrates such as Z-Phe-Arg-MCA, Z-Arg-Arg-MCA, Arg-MCA etc. were performed according to the procedure described by Barrett and Krischke (1981), incorporating slight modifications. A spectrofluorophotometer fitted with a thermostat regulated water bath was used for this study. The procedure is briefly described as follows:

Reagents:

i) Activator, Buffer: 340 mM sodium acetate containing 60 mM acetic acid and 4 mM disodium EDTA, pH 6.5. Freshly prepared dithiothreitol (8 mM) was added to the buffer on the day of its use.

ii) Substrate: 10 mM of the stock solution of the substrate in DMSO was made and stored below 0°C, until use. This stock was diluted to the required concentration with activator buffer without DTT.

iii) Diluent: Brij 35 (0.1 %) in water.

iv) Aminomethyl coumarin standard: A stock solution of 7-amino- 4-methyl coumarin (1mM) in DMSO was made and stored below 0°C. At the time of assay, the stock was diluted to the required concentrations with the activator buffer without DTT.

Procedure:

0.5 ml of the enzyme solution in diluent was incubated with 1.0 ml of activator buffer with 8.0 mM DTT, at 37°C for 5 min. The reaction was initiated by adding 0.5 ml of diluted substrate solution. The increase in fluorescence was monitored continously for 30 min by fixing excitation and emission wavelengths at 370 nm and 440 nm, respectively, using a suitable reagent blank for calibration. The total amount of 7-amino-4-methyl coumarin liberated during enzymatic assay was calculated from a standard curve of 7-amino-4-methyl coumarin prepared in a similar manner. One unit of enzyme activity was defined as the amount of enzyme required to release 1 μmol of 7-amino-4-methyl-coumarin per min.

***Colorimetric assay of cathepsin B with protein substrates*:**

Cathepsin B assay with acid denatured bovine milk casein, BSA and goat hemoglobin were done by the method described by Moore and Stein (1954) and Ahmad **et al.**, (1990).

After preparing a highly concentrated protein solution in the activator buffer (20 mM sodium phosphate buffer, pH 6.5, containing 2.0 mM each of EDTA and 2-mercaptoethanol), it was exposed to acetic acid at pH 2.8 and left overnight. The acid denatured protein was then kept in a boiling water bath for about 1 hr. After cooling to room temperature, it was centrifuged and filtered. The filtrate was taken and dialysed against the phosphate buffer till a pH of 6.5 was obtained. The final protein concentration of the filtrate was determined.

To 0.5 ml of the enzyme solution in activator buffer, preactivated at 37°C for 30 min, 0.5 ml of the substrate solution (as prepared above) was added and mixed properly. After 4 hr of incubation at 37°C, the reaction was terminated by adding 1 ml of chilled TCA (30% w/v) and the content was centrifuged for 10 min at 4000 rpm at 4°C. The clear supernatant thus obtained was collected. 1.0 ml of this supernatant (containing TCA soluble peptides and amino acids) was mixed with equal volume of freshly prepared ninhydrin reagent, and heated in a boiling water bath for 20 min. After cooling the solution under tap water, 5.0 ml of ethanol was added and the intensity of the blue colour formed was read at 570 nm against a suitable blank, prepared in the same way except that the protein-substrate was added after the addition of the TCA solution.

One unit of the enzyme was defined as the amount of enzyme required to increase the absorbance at 570 nm by 0.01 O.D. unit per hr under the above assay conditions.

Preparation of ninhydrin reagent:

5.0 gms of ninhydrin was solubilized in 125 ml of water at 90°C. To this solution 25 ml of ascorbic acid solution (20%) was gradually added and the reaction was allowed to occur for 30 min, at room temperature. The crystals of hydrindatin thus formed were excessively washed with chilled water and dried at 37°C. 0.3 gm of hydrindantin and 2.0 gms of ninhydrin were dissolved in 4 M sodium acetate buffer, pH 5.5, to a final volume of 25 ml. 75 ml of methyl cellosolve was then added to it, and after shaking the mixture thoroughly, the reagent was stored at 4°C in a brown bottle for future use.

***Determination of catalytic Parameters of Cathepsin B*:**

The values of K_m and V_{max} of the purified goat spleen cathepsin B for synthetic as well as protein substrates were calculated from the least squares analysis of the data plotted according to the method of Lineweaver and Burk (1934) using the general equation:

$$1/V = K_m/V_{max} (1/[S]) + 1/V_{max}$$

The substrate concentrations were chosen on the assumption that the initial velocity of the enzyme-reaction provided the accurate values for the K_m when the substrate concentration ranges between 20-80% saturation.

9. Chemical analysis :

a) End group analyses of cathepsin B :

i) Identific ation of NH_2-terminal residue: Determination of the NH_2-terminal amino acid residue of the purified cathepsin B from goat spleen was done according to the method of Gray (1967).

To 1 ml of the protein solution (containing 32 µg of cathepsin B), urea and sodium bicarbonate were added to a final concentration of 8 M and 0.5 M, respectively and left at 37°C for 1 hr. 1 ml dansyl chloride (20 mg/ml of acetone) was then added to the above mixture containing the protein, and incubated at 37°C overnight. The whole content was then dialysed thoroughly against distilled water so as to remove the free dansyl chloride, salts and other low molecular weight substances.

The dansylated protein was taken in a tube with 5.7 N freshly distilled HCl. The open end of the tube was sealed and the protein hydrolysis was performed at 105-110°C for 20 hr in a thermo reacti-therm (Pierce Chem. Co.). The content was evaporated to dryness and the residue was dissolved in 50% aqueous pyridine solution. Identification of the dansylated amino acid was finally done with thin layer chromatography (TLC) on polyamide sheets.

ii) Identification of COOH-terminal residue: The COOH-terminal amino acid residue of the purified goat spleen cathepsin B was identified as described by Narita (1970). The procedure is briefly described below:

To 2 ml of cathepsin B (0.36 mg) solution in 60 mM sodium phosphate buffer, pH 8.0, 0.72 gm urea, 30 ml 2-mercaptoethanol and 18 mg iodoacetamide were added in such a way that the final concentration of urea, 2-mercaptoethenol and iodoacetamide were 6 M, 1% and 0.05 M, respectively. The reaction mixture was incubated at 37°C overnight followed by extensive dialysis against 60 mM sodium phosphate buffer, pH 8.0, containing 6.0 M Urea.

A solution of carboxypeptidase A was prepared by suspending 5.0 mg (equivalent to 250 I.U.) of the enzyme in 5.0 ml of water. The suspension was centrifuged and the supernatant obtained was discarded. The residue was collected and placed in an ice bath for 5-6 min prior to addition of 0.1 ml (1%) sodium bicarbonate. 1 N NaOH was gradually added to it so that all the enzyme crystals dissolved. The final pH of this enzyme solution was carefully adjusted to pH 8.0 by titrating against 0.1 N HCl. The concentration of the enzyme solution was determined spectrophotometrically using the extinction coefficient of 8.6×10^4 (Neurath, 1955). The molecular weight of the enzyme was taken to be 34 kDa (Narita, 1970). The enzyme solution was finally diluted to a concentration of 1 mg/ml with 60 mM sodium phosphate buffer of pH 8.0 containing 6.0 M urea.

The enzymatic reaction was performed at room temperature by taking the solution of cathepsin B and that of carboxypeptidase A in a molar ratio of 1:2. One ml aliquots were taken out of the reaction mixture at various time intervals (eg., 0, 5, 10, 20, 30, 60, and 120 min) and the reaction was stopped by lowering the pH by addition of 1.0 ml of 1.0 N HCl. Control was prepared in the same way except that the HCl was added to the enzyme solution before addition of substrate (Cathepsin B). The acid precipitated proteins were removed by centifugation and the supernatent thus obtained was analysed for the liberated amino acid(s) by TLC after dansylation.

b) Determination of Free Thiol group(s) :

The free sulfhydryl content of the purified cathepsin B, both under native as well as denatured conditions (8.0 M urea) were performed according to the procedure of Ellman (1959).

Ellman's reagent was prepared by dissolving 10 mM 5,5'-dithiobis (2-nitrobenzoic acid), (DTNB), in 0.1 M phosphate buffer, pH 7.9, containing 0.1 mM EDTA. The solution was flushed with nitrogen before use. 0.2 ml of this reagent was added to 3.0 ml of nitrogen flushed enzyme solution (prepared by mixing 0.3 ml of enzyme solution and 2.7 ml of phosphate buffer decribed above with or without 8.0 M urea) and the absorbance at 412 nm was continously monitored for about 30 min till no further increase

was observed. The sulfhydryl content of the protein was calculated by using the equation.

$$C_0 = A \times D/E$$

Where C_0 was the concentration of the thiol group(s), A, the absorbance at 412 nm, E, the extinction coefficient of the coloured complex (13600/cm/M) and D, the dilution factor.

c) Determination of tryptophan residues :

Total tryptophan residues of cathepsin B was determined colorimetrically by the method of Spies and Chambers (1949) using p-dimethyl aminobenzaldehyde (DAB).

To 0.5 ml of salt-free enzyme solution (containing known amount of protein), 4.0 ml of 23.8 N H_2SO_4 was added and mixed thoroughly. 0.5 ml of freshly prepared DAB solution (30 mg/ml in 2.0 N H_2SO_4) was then added to it and mixed well. The reaction was carried out for 18 hr at room temperature in the dark. Following this, 0.1 ml of freshly prepared aqueous solution of 0.045% $NaNO_2$ was added to the reaction mixture and mixed properly. After incubating further for 60 min in the dark, the absorption of the solution was measured at 590 nm against a suitable reagent blank. The tryptophan content was calculated from the standard curve prepared in the similar way with standard tryptophan.

d) Determination of carbohydrate content :

The total carbohydrate content of the purified cathepsin B was determined by phenol-sulphuric acid reaction using the standrad method of DuBois **et al.**, (1956). The total sugar content was expressed in terms of D-glucose equivalent determined with the help of a standrad curve prepared with glucose solution.

To 1.0 ml of the solution (containing varying amounts of cathepsin B) 1.0 ml of 5% (w/v) phenol solution was added and mixed thoroughly. Finally 5.0 ml concentrated sulphuric acid as added carefully and the reaction mixture shaken properly. The colour was allowed to develop at room

temperature, and the absorbance of the brown colour thus obtained was measured at 485 nm against a suitable reagent blank.

10. Amino acid analysis :

Amino acid analysis of the purified cathepsin B was done by using high performance liquid chromatography (HPLC) using a single Na^+ type cation exchange column (ISC-07/S1504 Na^+) form Shimadzu Co. The following buffers were used:

I. Sample buffer : 0.2 M sodium phosphate buffer pH 2.2, containing 7% v/v ethanol. The pH was adjusted with 80% perchloric acid.

II. Solution A : 0.2 M sodium citrate buffer, pH 3.2 containing 7% v/v ethanol and 1% (v/v) perchloric acid.

III. Solution B : 0.6 M sodium citrate buffer, pH 10.6 containing 1.24% (w/v) boric acid and 3% 4 M sodium hydroxide solution.

IV. Solution C : 0.2 M sodium hydroxide solution.

V. Reaction reagent : Sodium carbonate (0.348 M), boric acid (0.126 M) and potassium sulfate (0.108 M), dissolved in distilled water.

VI. OPA Solution : 200 mg o-pthalaldehyde (OPA) was dissolved completely in 3.5 ml of ethanol followed by addition of 250 mg N-acetyl-L-cysteine and 1.0 ml of 10% Brij 35 solution. A final volume of 250 ml with distilled water was made.

VII. Sodium hypochlorite solution : 50 ml of sodium hypochlorite/ 250 ml reaction mixture.

***Preparation of sample for HPLC analysis*:**

In a hydrolysis tube (Pierce Chem. Co.) 0.3 ml of protein solution (containing about 4.0 nmols of purified cathepsin B) was taken and dried under vacuum. 0.5 ml of freshly distilled 5.7 N HCl was added and sealed properly. Hydrolysis was performed at 110°C for 6, 12, 18 and 24 hrs after which HCl was evaporated by passing a stream of nitrogen through the protein-HCl solution. After the HCl had completely evaporated 100 µl of

HPLC sample buffer, pH 2.2 (stated above) was added to the hydrolysate. The content was then centrifuged and finally filtered through a millipore filter (0.45 μM) before injecting onto the HPLC column.

11. Determination of specific extinction coefficient :

The protein solution was extensively dialysed against water and passed through a column of mixed-bed resin of Amberlite MB-3. The pH of the eluent containing the protein was then directly measured to get its isoionic pH. The absorbance of the protein solution thus obtained was measured at 280 nm. Known volumes of this solution were taken in different pre-weighed weighing bottles which were then heated to dryness at 105-110°C. The bottles were weighed repeatedly (after heating and cooling each time) at fixed intervals of time until a constant weight was obtained. The weights of the empty bottles were substracted from that of the respective bottles containing protein, to give the actual weight of the protein taken in a given weighing bottle. The specific extinction coefficient ($E^{1\%}_{1cm}$) of the protein was calculated by dividing the optical density of the protein solution by its weight (gm/ 100 ml).

12. Measurement of intrinsic viscosity :

The viscosity of the purified goat spleen cathepsin B was measured in a Schott Gerate (Type 513 00) viscometer having a flow time of about 430 sec for 3 ml water at 25°C.

After cleaning the viscometer properly, it was dipped in an insulated glass water-bath fitted with a thermostat (HAAKE, model D8) in which the temperature was maintained within $\pm$ 0.05°C. 3.0 ml of protein solution (previously dialysed in appropriate buffer and passed through millipore filter (pore size 0.45 μM) of varying concentrations (0-2 mg/ml) were poured in the viscometer and the time of fall of the enzyme solution (t), and that of the solvent i.e. buffer (t_o), were recorded with the help of a stop watch having a least count of 0.1 sec. The intrinsic viscosity [η] of the protein solution was calculated by the method of Tanford (1955) using the following expression :

$$[\eta] = \lim_{c \to 0} (\eta - \eta_o)/\eta c$$

$$= \lim_{c \to 0} [(t-t_o)/t_o c] + (1-\bar{v}_2 \rho_o) / \rho_o$$

where, η_o and η were the viscosities in poise of the solvent and protein solution respectively, c was the protein concentration in gm/ml, ρ_o was the density of the solvent in gm/ml and $\bar{v}_2$ the partial specific volume of the protein in ml/gm.

13. Immunological studies :

Polyclonal antibodies were raised in rabbits of 2-3 kgs body weight. The animals were immunized by administering 140 µg of cathepsin B with Fruend's Complete Adjuent (FCA) subcutaneously followed by a booster dose given at 30 day intervals with 70 µg of the enzyme with Fruend's Incomplete Adjuent (IFA). Sera was collected 4 days after giving the booster dose. The cross-reactivity of the antisera with cathepsin B was checked by Ouchterlony double diffusion (Ouchterlony, 1949).

Briefly, 1.2% (w/v) agarose solution in water, containing 0.02% sodium azide, was prepared by boiling it for 5-10 min. The agarose solution was uniformly layered on glass slides and was allowed to cool down to room temperature. After gelation of agarose on the slides, wells were made. The central well was filled with the antisera. The cross reaction was allowed to occur at 37°C overnight and the slides were examined for the formation of precipitin arcs, if any. The slides were finally stained with 0.1% amido black solution for 10 min at room temperature. Destaining was done by mechanical shaking with 7% acetic acid in 5% methanol.

RESULTS

A. Isolation and purification of cathepsin B :

Cathepsin B was isolated from goat spleen by modifying our earlier procedure (Ahmad **et al.**,1989). The modification, which also included an additional step involving CM-Sephadex chromotography, significantly improved the degree of purity of the enzyme. The protein fraction precipitating between 40-75% of ammonium sulfate saturation was subjected to gel filtration on a Sephadex G-75 column. The elution profile thus obtained is shown in Fig. 2. The fractions containing significant amount of BANA hydrolase activity were combined and subjected to ion-exchange chromotography on DEAE-Cellulose column. The unbound protein fractions had appreciable activity against the substrate Leu-NA, thus showing the presence of cathepsin H. The bound protein fractions which eluted with 0.2 M sodium chloride in the equilibrating buffer, showed significant amount of BANA hydrolase activity but no activity was obtained against Leu-NA (Fig. 3). Thus, these active fractions of the enzyme were pooled and subjected to ion-exchange chromatography on a CM-Sephadex cation exchanger column. Fractions eluting with 20 mM sodium acetate buffer, pH 5.6, showed appreciable BANA and Z-Arg-Arg-MCA hydrolase activity, characteristic of cathepsin B (Fig. 4).

The polyacrylamide gel electrophoresis of the enzyme preparation thus obtained, on 7.5% gel at pH 8.3 resulted in two protein bands. This was termed as unresolved goat spleen cathepsin B (CB) (Fig. 6(a)). In an attempt to resolve the two bands, the enzyme preparation was further subjected to affinity chromatography on a ConA-Sepharose 4B column following the procedure described by Takahashi **et al.**, 1986a. Two distinct enzymatically active protein peaks were obtained which were designated as goat spleen cathepsin B-I (GSCB-I) and goat spleen cathepsin B-II (GSCB-II) (Fig. 5 & Fig. 6(b)&(c)). GSCB-I did not bind to the ConA-Sepharose column. The bound GSCB-II was eluted with 0.5 M methyl-α-D-glucopyranoside.

Fig.2 Elution profile of goat spleen cathepsin B through Sephadex G-75 column. About 120 mg of protein was applied on the column (2.6x90cm) equilibrated with 50 mM sodium acetate buffer, pH 5.0, containing 1 mM EDTA and 0.02%. sodium azide. 5.0 ml fractions were collected and monitored for proteins at 280 nm (●—●) and for cathepsin B activity using BANA as substrate, at 540 nm (✱—✱). Horizontal bar indicates the active fractions pooled for further purification.

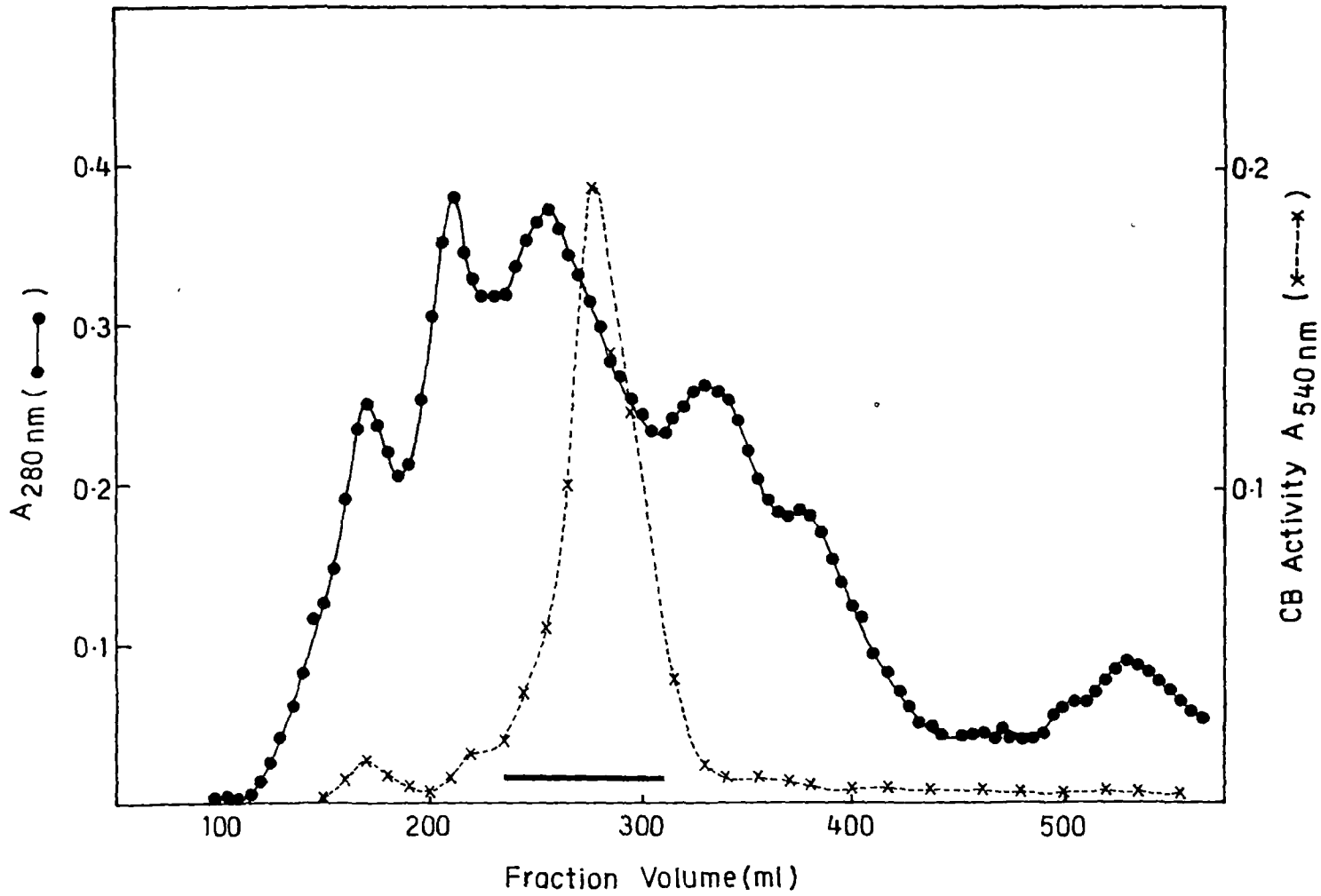

0·4
0·3
0·2
0·1
$A_{280\,nm}$ (●—●)
0·2
0·1
CB Activity $A_{540\,nm}$ (×---×)
100
200
300
400
500
Fraction Volume (ml)

Fig.3 Elution profile of cathepsin B active fractions through DEAE-Cellulose chromatography.

About 50 mg of protein was applied onto the column (1.6x14 cm) equilibrated with 0.02 M tris-acetate buffer, pH 6.0, containing 1 mM EDTA and 0.02% sodium azide. 10 ml fractions at a flow rate of 40ml/hr were collected and were monitored for proteins at 280 nm (●—●). Cathepsin B activity was determined using BANA (✱---✱) and Leu-NA (□—□) as substrates. Horizontal bar indicates the pooled cathepsin B active fractions which were subjected to further purification.

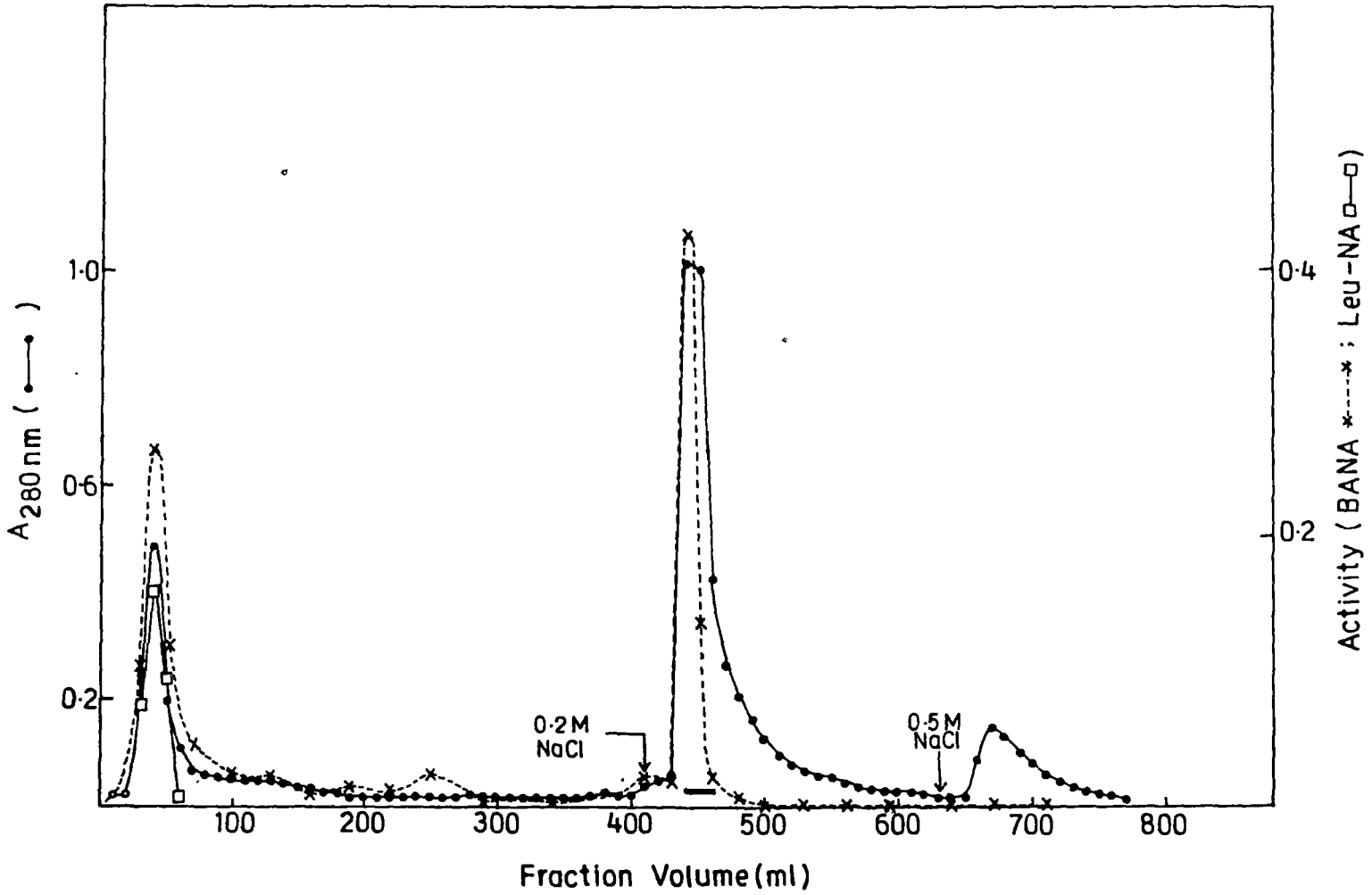

1·0
0·6
0·2
$A_{280\,nm}$ (—●—)
0·4
0·2
Activity (BANA *----*----* ; Leu-NA □—□—□)
0·2 M NaCl
0·5 M NaCl
100
200
300
400
500
600
700
800
Fraction Volume (ml)

Fig.4 Elution profile of cathepsin B (obtained through DEAE-Cellulose chromatography) from CM-Sephadex cation exchange column. About 40 mg of protein was applied onto the column (1.6x12 cm) pre-equilibrated with 20 mM sodium acetate buffer, pH 4.8, containing 1 mM EDTA and 1.4 mM 2-mercaptoethanol. The column was washed with the same buffer at pH 4.8, followed by step wise elution at pH 5.6, 6.0 and finally using 1.0 M NaCl in the buffer at pH 6.0. Activity was determined using BANA (⁎--⁎) as substrate. Horizontal bar shows the active cathepsin B fractions pooled.

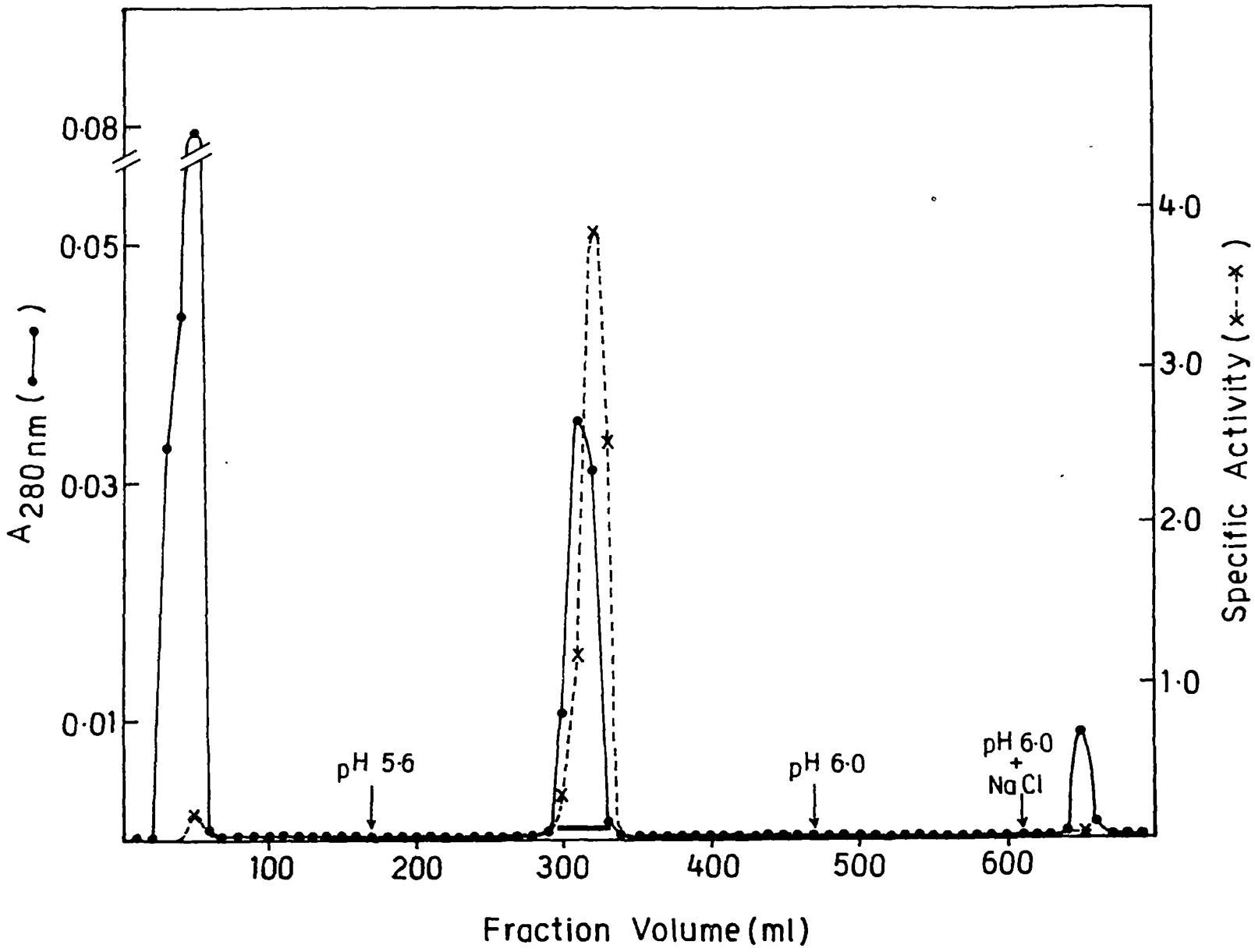

0·08
0·05
0·03
0·01
A280nm (—●—)
4·0
3·0
2·0
1·0
Specific Activity (×--×)
pH 5·6
pH 6·0
pH 6·0
+
NaCl
100
200
300
400
500
600
Fraction Volume (ml)

Fig.5 Separation of cathepsin B on a ConA-Sephasose 4B column.
Purified cathepsin B preparation was applied on a ConA-Sepharose 4B column (2.5x6.0 cm) equilibrated in 0.05 M sodium acetate buffer, pH 6.0, containing 0.2 M NaCl , 1.43 mM 2-mercaptoethanol and 1 mM each of $CaCl_2$ and $MnCl_2$. A major peak of cathepsin B (GSCB-I) appeared in the washing fractions. However, when the column was extensively washed with the same buffer and then with 0.5 M methyl glucose (indicated by the arrow) the retained peak of cathepsin B (GSCB-II) was eluted. The enzyme activities were assayed with Z-Arg-Arg-MCA and the active fractions (shown by bars) were pooled .

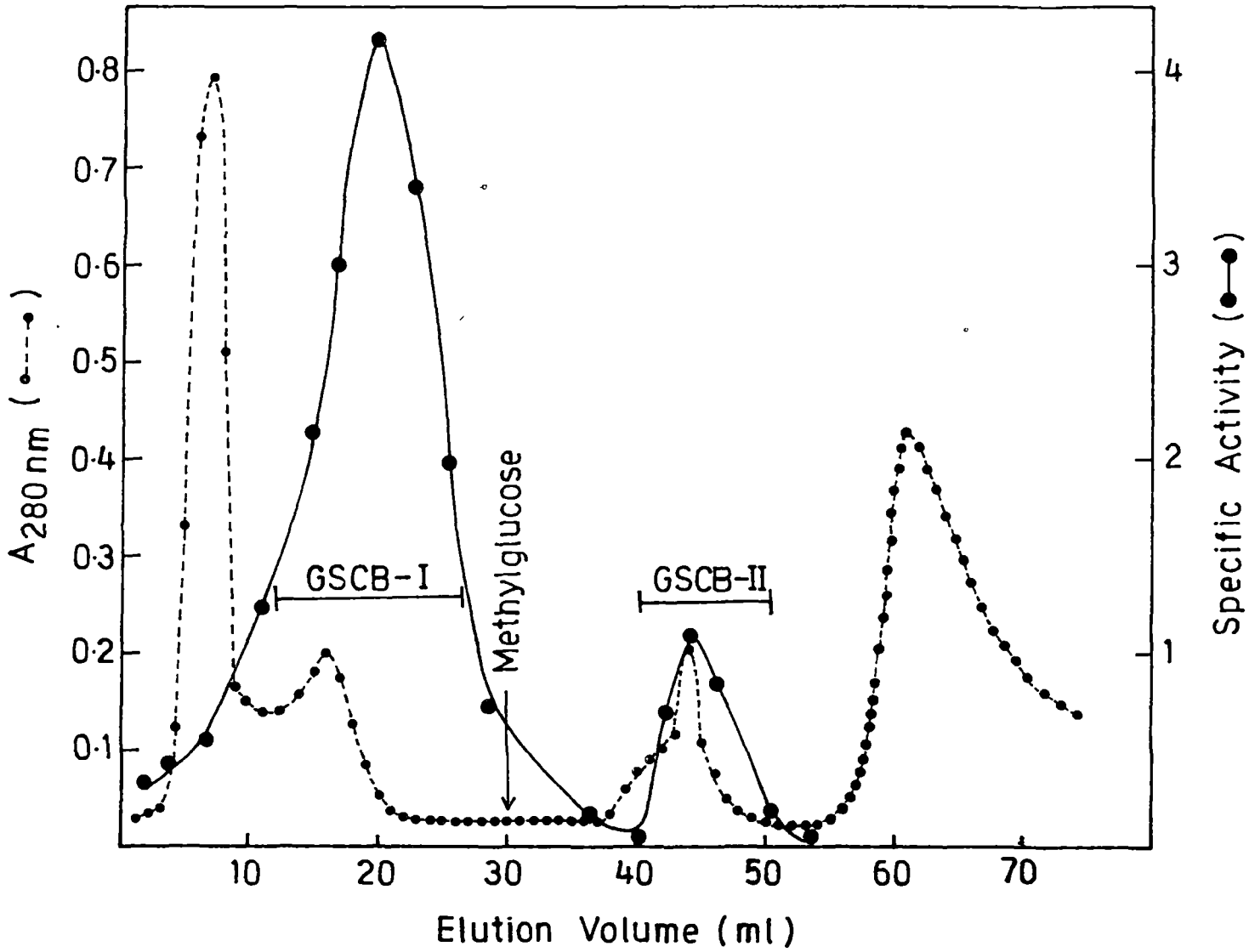

0·8
0·7
0·6
0·5
0·4
0·3
0·2
0·1
A280 nm (•---•)
4
3
2
1
Specific Activity (•—•)
GSCB-I
Methylglucose
GSCB-II
10
20
30
40
50
60
70
Elution Volume (ml)

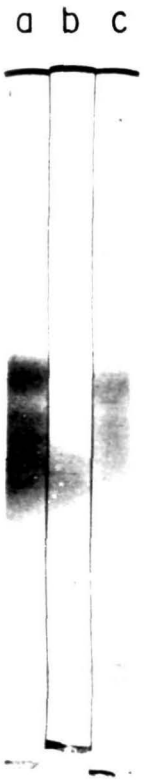
TABLE I : Purification of cathepsin B from goat spleen

Steps	Total Protein (mg)	Total activity (U)	Specific activity (U/mg protein)	Purification fold	Yield (%)
Crude extract	28085	612	0.0218	1	100
Acid extraction	1530	282	0 184	8.44	46
40-75% $(NH_4)_2SO_4$ precipitate	168	141	0.84	38.53	23
Sephadex G-75	34.6	57.5	1.66	76.15	9.4
DEAE-Cellulose	11.8	29.4	2.49	114.22	4.8
CM-Sephadex*	3.6	13.9	3.86	177.06	2.3
Con-A Sepharose 4B					
GSCB-I	2.6	10.8	4.16	190.82	1.76
GSCB-II	1.98	2.1	1.06	48.62	0.34

Cathepsin B was purified as described in materials and methods section. Determination of enzyme activity and unit expression is as mentioned in methods section. GSCB-I, goat spleen cathepsin B-I; GSCB-II, goat spleen cathepsin B-II

* Cathepsin B preparation after CM-Sephadex step is termed as pruified unresolved cathepsin B.

Fig.6 Polyacrylamide gel electrophoretic pattern of the purified unresolved goat spleen cathepsin B(a), goat spleen cathepsin B-I(b) and goat spleen cathepsin B-II (c).
Approxmately 4-6 μg of protein was applied on the 7.5% gel and electrophoresced for about 1 hr with a current of 2 mA per tube. The gels were stained in Coomassie Brilliant Blue and destained with a mixture of 7% acetic acid and 5% methonal.


a b c

GSCB-I constituted approximately 84% of the total enzyme activity and had a specific activity of 4.16 units/mg of protein towards the substrate Z-Arg-Arg-MCA, whereas GSCB-II which constituted about 16% of the total activity had a specific activity of 1.06 units/mg of protein.

Most of the studies were performed using GSCB-I (unless stated otherwise) as its yield as well as specific activity was higher compared to GSCB-II. However, comparative studies of both GSCB-I & II were done in order to show that these were two probable isozymes.

The methodology for the purification developed here is simple and provided enzyme with high specific activity and relatively high yield.

B. Characterization of cathepsin B :

a) Molecular weight determination:

i) Gel filtration:

The molecular weightsof cathepsin B (GSCB-I & II) were determined by gel chromatography on a Sephadex G-100 column (1.6x65cm) calibrated with five standard molecular weight marker proteins (Fig. 7). The elution profile of GSCB-I & II are shown in Fig. 8.

The void volume, V_o, of the column was 44 ml as determined by passing Blue Dextran 2000 through the column. The elution volume of $K_3Fe(CN)_6$, V_p, was 128 ml.

The total volume, V_t of the column, computed using the relation $V_t = \pi r^2 l$, where r, is the radius and l, the length of the column, was 131 ml. The elution volume, V_e, of the different marker proteins as well as GSCB-I & II were determined from their elution profiles. The distribution coefficient, K_d and the available distribution coefficient, K_{av} were calculated with the help of the following equations :

$$K_d = (V_e - V_o) / V_i$$

$$K_{av} = (V_e - V_o / V_t - V_o)$$

Fig.7 Gel filtration chromatography of various marker proteins on a calibrated Sephadex G-75 column.

About 5-10 mg of each standard marker protein was applied to the column equilibrated with 60 mM sodium phosphate buffer, pH 6.0. The markers applied were (1) BSA (67 kDa),(2) ovalbumin (43 kDa), (3) α-chymotrypsinogen (25.7 kDa), (4) myoglobin (17.8 kDa) and (5) cytochrome c (12.4 kDa).

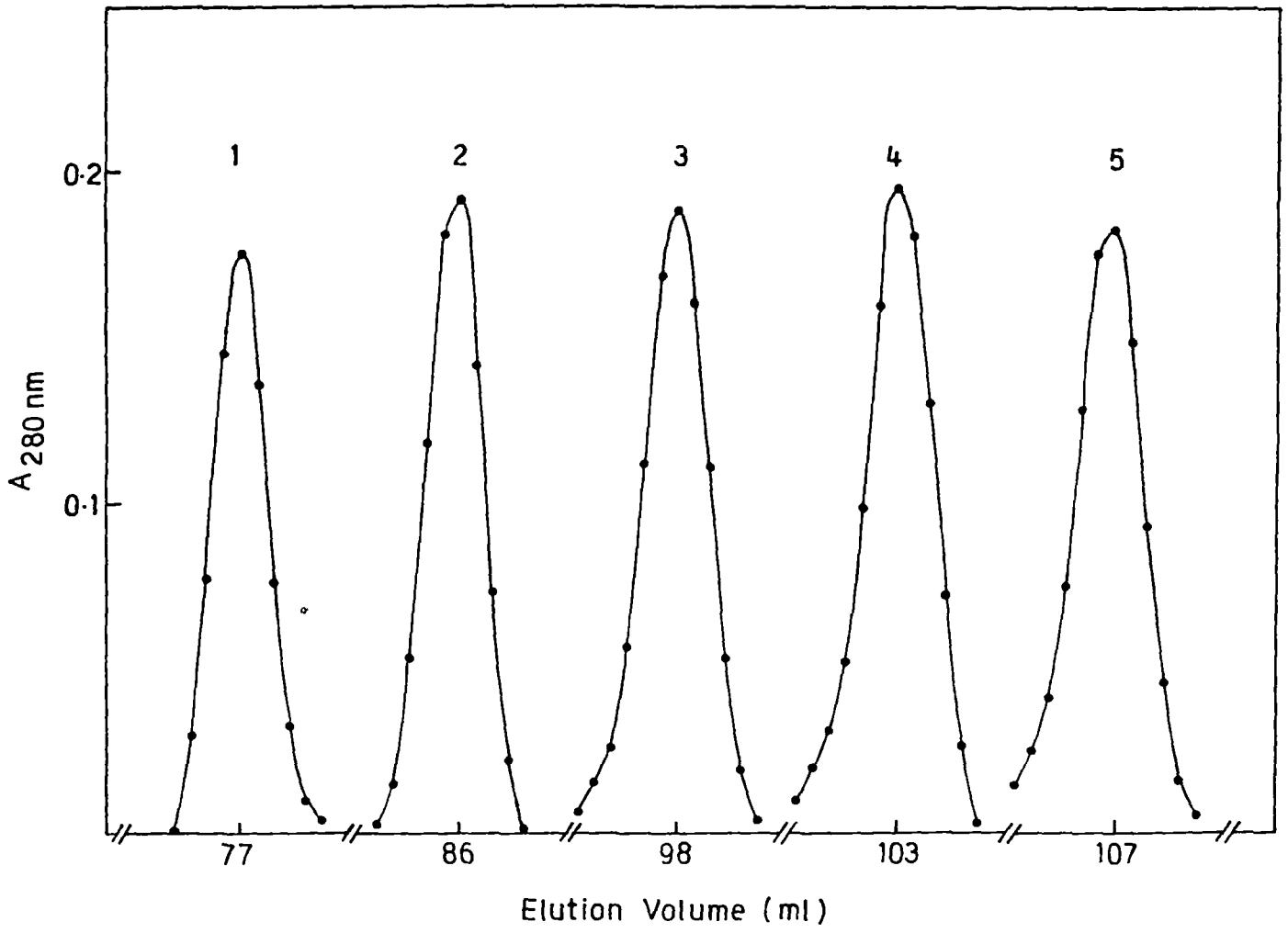
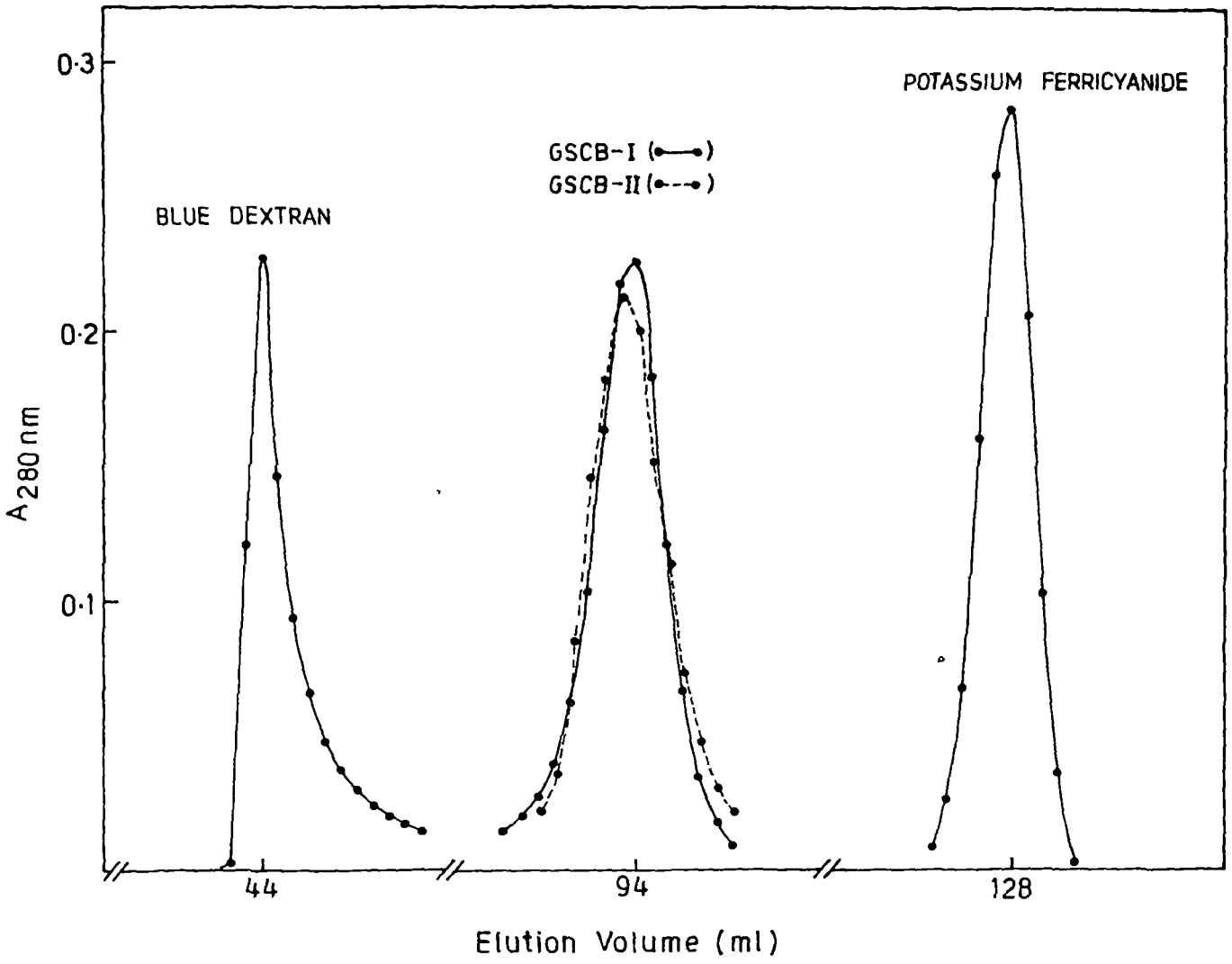

1
2
3
4
5
0·2
0·1
$A_{280\,nm}$
77
86
98
103
107
Elution Volume (ml)

Fig.8 Gel filtration chromatography of Blue Dextran 2000, goat spleen cathepsin B-I & II and postasium ferricyanide ($K_3Fe(CN)_6$) through a calibrated Sephadex G-75 column.


0·3
0·2
0·1
$A_{280\,nm}$
BLUE DEXTRAN
GSCB-I (•—•)
GSCB-II (•---•)
POTASSIUM FERRICYANIDE
44
94
128
Elution Volume (ml)

where V_t and V_i are the total and the inner volumes of the column, respectively.

The values of K_d and K_{av}, obtained by theoretical treatment of data by Andrews (1970) and Porath (1963), for GSCB-I were found to be 0.5952 and 0.5517 respectively and for GSCB-II the values were 0.5714 and 0.5747 respectively, table II. The average molecular weights of GSCB-1 & II was thus found to be approximately 28.1 kDa & 31.6 kDa respectively (table III; Fig. 9 & 10).

ii) Sodium Dodecyl Sulfate-Polyacrylamide Gel Electrophoresis:

Molecular weights of GSCB-I & II were also determined by sodium dodecyl sulfate-polyacrylamide gel electrophoresis on 12.5% polyacrylamide gels containing 1.5% SDS (Fig. 12). Using the relative mobilities, R_m, of the different standard marker proteins, a plot of R_m against their corresponding log of molecular weights gave a straight line, yeilding molecular weight of about 25.7 kDa and 26.6 kDa for GSCB-I & II, respectively (Fig. 12; table IV). Both of them showed single band on SDS-PAGE, in the presence and absence of 2-mercaptoethanol indicating single chain forms.

The molecular weights determined both by gel filtration and SDS-PAGE, was thus comparable to the values (23-29 kDa) obtained for cathepsin B as reported earlier from other sources.

The fact that the values of molecular weights obtained by SDS-PAGE is lower than the values obtained by gel-filtration for GSCB-I & II, indicate that they are hydrated under native conditions.

b) Hydrodynamic Properties :

i) Determination of Stokes radius and frictional ratio :

Stokes radii of GSCB-I & II were determined from the data obtained by analytical gel filtration, according to the method of Laurent and Killander (1964). The results shown in table III, Fig. 14, yielded a value of 24.4 and 25.8 nm for GSCB-I & II respectively.

TABLE - II : Hydrodynamic parameters determined from analytical gel filtration of standard marker proteins and goat spleen cathepsin B (GSCB-I & II).

Protein	V_e/V_o	K_d	$K_d^{1/3}$	K_{av}	$(-\log K_{av})^{1/2}$
Bovine serum albumin (BSA)	1.750	0.393	0.732	0.379	0.649
Ovalbumin	1.955	0.500	0.794	0.483	0.562
α-Chymotrypsinogen	2.227	0.643	0.863	0.621	0.455
Cytochrome C	2.432	0.750	0.909	0.724	0.374
Myoglobin	2.341	0.702	0.889	0.678	0.411
Cathepsin B					
(GSCB-I)	2.136	0.595	0.841	0.552	0.491
(GSCB-II)	2.091	0.571	0.830	0.575	0.508

TABLE - III : Molecular weight and Stokes radii of marker proteins and goat spleen cathepsin B (GSCB-I & II).

Protein	Molecular Weight (kDa)	Stokes radius (Å)
Bovine serum albumin (BSA)	67.0[a]	35
Ovalbumin	43.6[b]	30
α-Chymotrypsinogen	25.7[a]	22
Myoglobin	17.8[a]	19
Cytochrome C	12.4[c]	17
Cathepsin B		
(GSCB-I)	28.1[d]	24.6
(GSCB-II)	31.6[d]	25.8

a : Tanford **et al.**, (1968)
b : Castellino and Barker (1968)
c : Eck and Dayoff (1966)
d : Determined in the present study.

Fig.9 Plot of elution volume/void volume (V_e/V_0) versus logarithm of molecular weight (log M).
The marker proteins are same as given in the legend to Fig.7. The straight line was drawn by the method of least squares which fits the equation $V_e/V_0 = 6.47 - 0.98 \log M$.

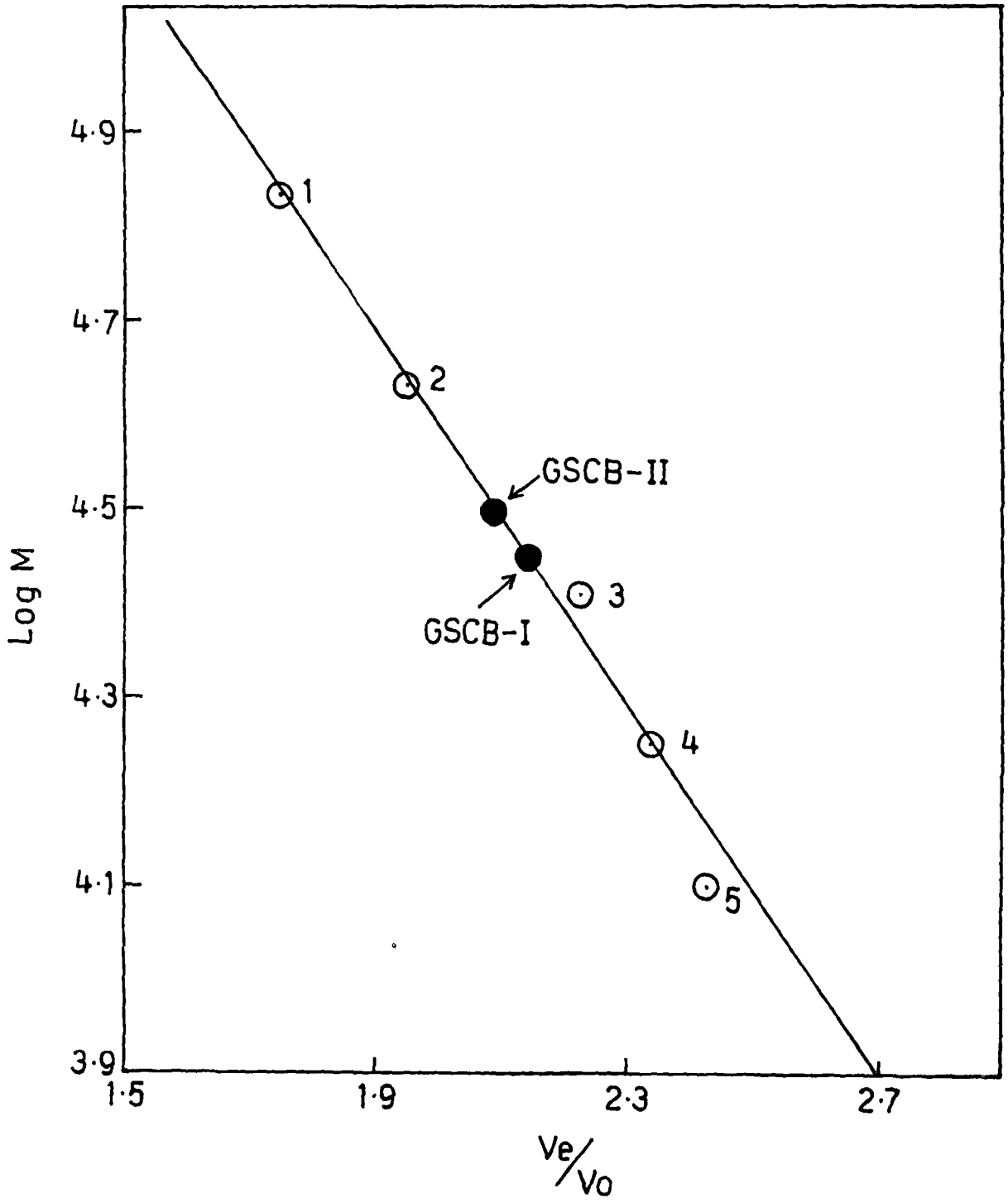

4·9
4·7
4·5
4·3
4·1
3·9
Log M
1·5
1·9
2·3
2·7
Ve/Vo
1
2
3
4
5
GSCB-II
GSCB-I

Fig.10 Porath plot for determination of molecular weight of goat spleen cathepsin B.

The maker proteins from 1-5 are same as described in the legend to Fig.7.. The linear plot obtained by the method of least squares, fits the equation. $M^{1/3} = 110.62 - 95.22\ K_d^{1/3}$.

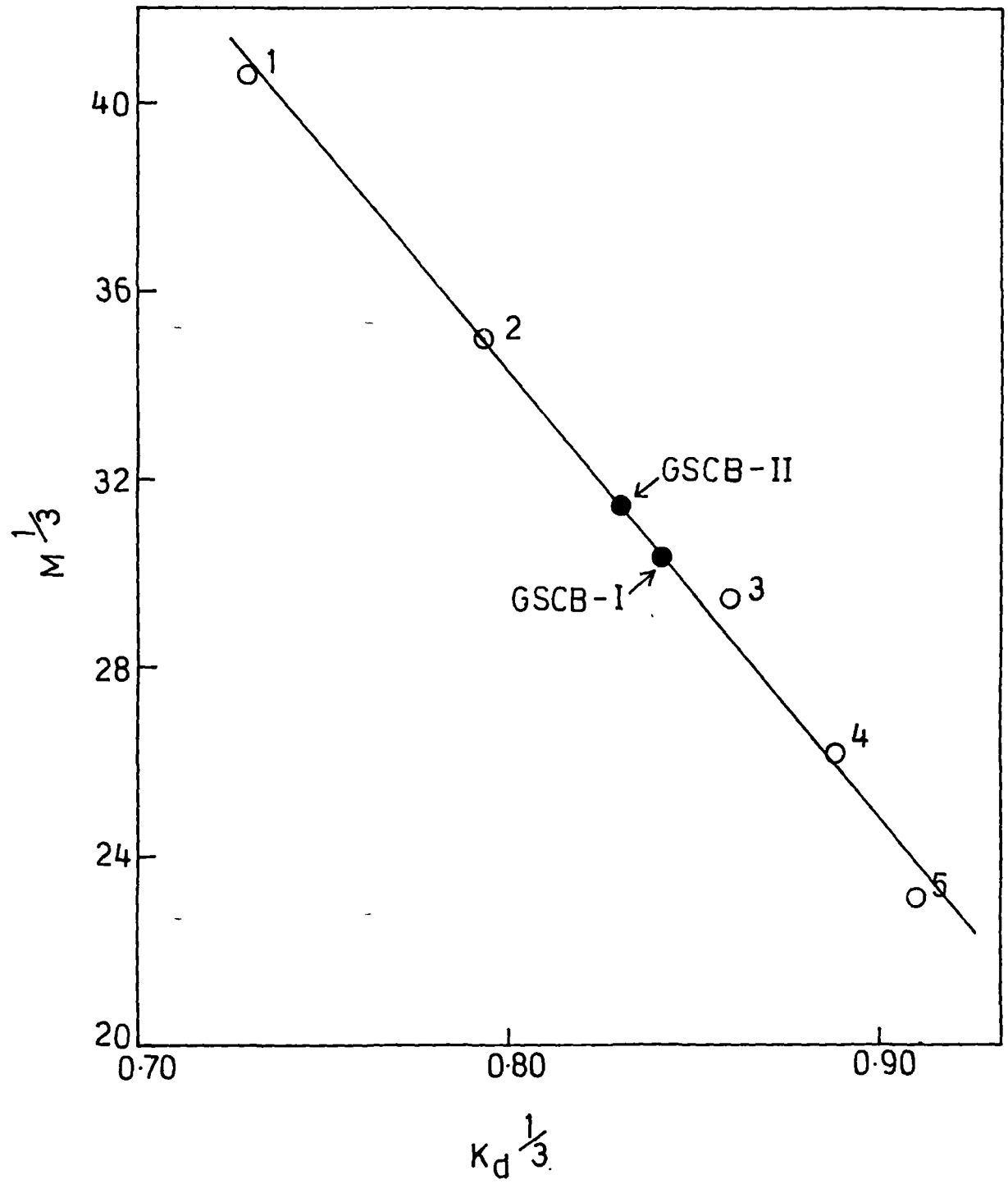

40
36
32
28
24
20
0·70
0·80
0·90
M 1/3
Kd 1/3
1
2
3
4
5
GSCB-II
GSCB-I

Fig.11 SDS-PAGE (12.5%) of unresolved goat spleen cathepsin B. The standard molecular weight marker proteins are (1) BSA (67 kDa), (2) ovalbumin (43 kDa), (3) α-chymotrypsinogen (25.7 kDa), (4) myoglobin (17.8 kDa) and (5) cytochrome c (12.4 kDa). 8-10 μg of purified protein was electrophoresed at pH 8.3, I=0.02 with a current of 3 mA per tube.

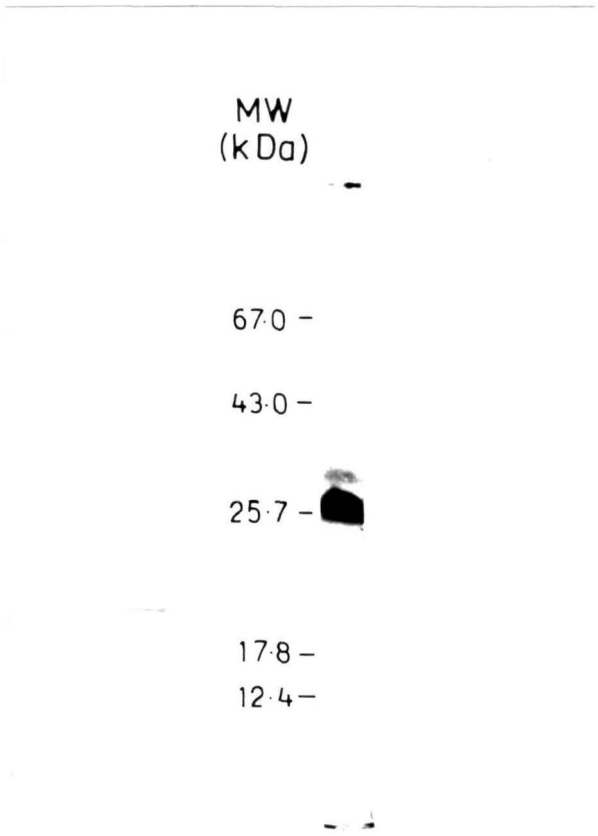

MW
(kDa)
67·0
43·0
25·7
17·8
12·4

Fig.12 SDS-PAGE pattern of GSCB-II(a) and GSCB-I(b) on 12.5% gels. 8-10 μg of both the protein fractions were electrophoresed at pH 8.3, I=0.02 with a current of 3mA per tube. Arrow indicates the position of tracking dye.


a
b

Fig.13 Determination of molecular weights of goat spleen cathepsin B-I & II using relative mobilities (R_m) on SDS-PAGE.
The marker protiens from 1-5 were the same as described in the legend to Fig.11. The straight line was plotted according to least squares method which follows the equation log M = 5.058 - 1.153 R_m.

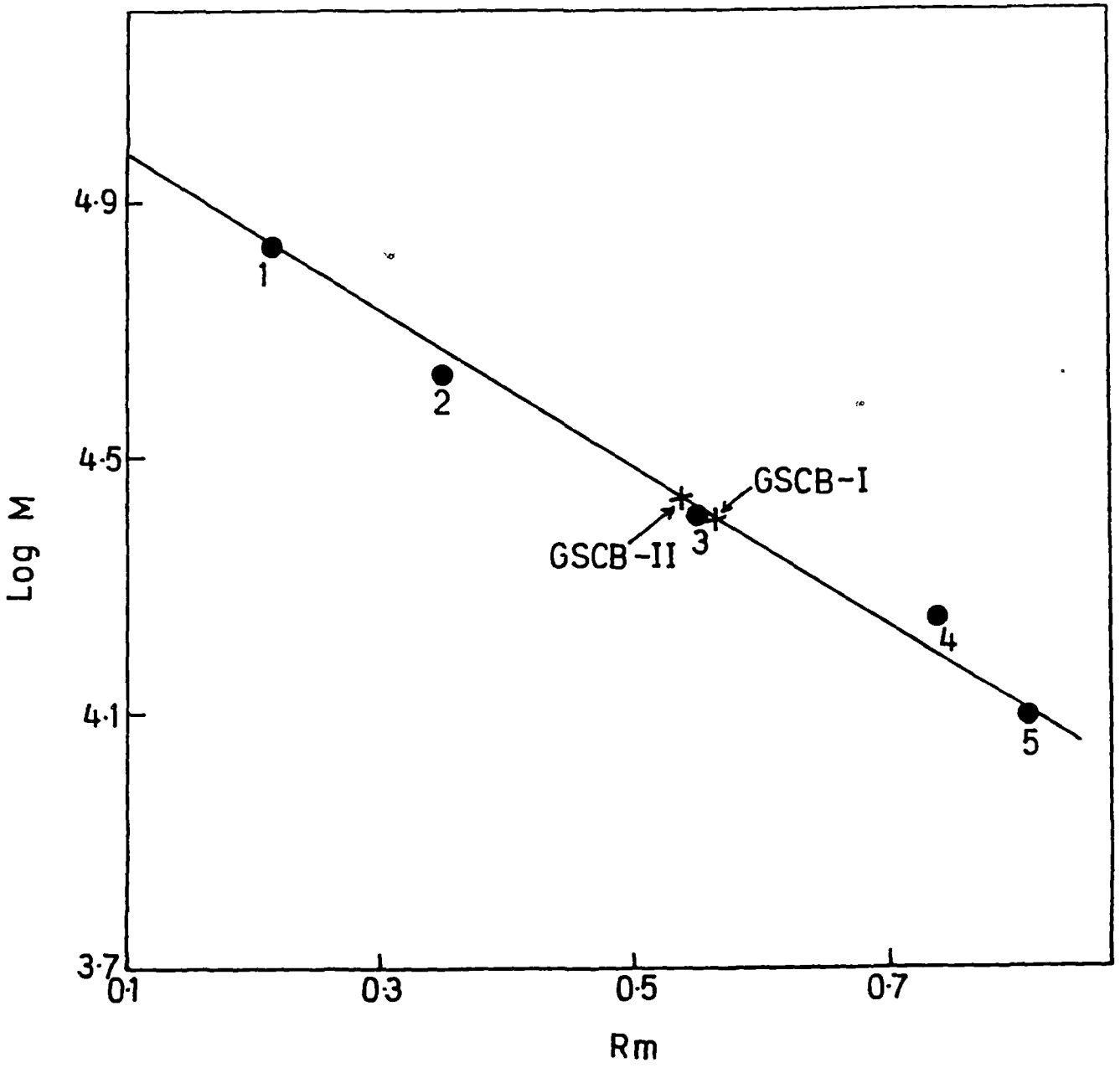

4·9
4·5
4·1
3·7
Log M
0·1
0·3
0·5
0·7
Rm
1
2
3
4
5
GSCB-I
GSCB-II

Fig.14 Determination of Stokes radius of goat spleen cathepsin B-I & II. The marker proteins from 1-5 were the same as described in the legend to Fig.7. The straight line was drawn by the method of least squares which fits the equation, $(-\log K_{av})^{1/2} = 0.015r + 0.125$ where K_{av} represents available distribution coefficient and r the relative mobility. Details of the procedure is described in the experimental section.

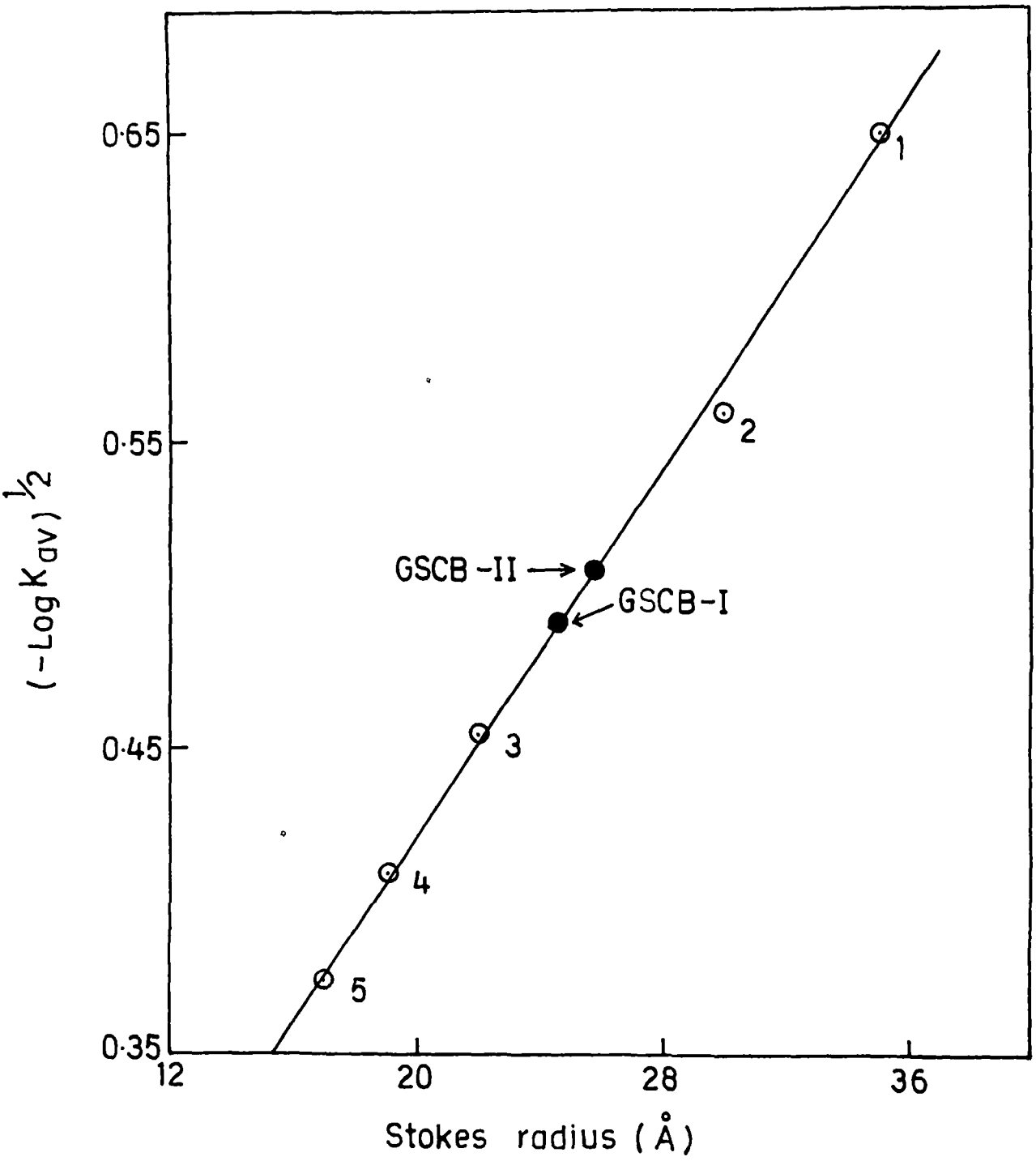

0·65
0·55
0·45
0·35
$(-\text{Log}\, K_{av})^{1/2}$
12
20
28
36
Stokes radius (Å)
1
2
3
4
5
GSCB-II
GSCB-I

TABLE - IV : Molecular weight (M) and relative mobility (R_m) values of marker proteins and goat spleen cathepsin B (GSCB-I and II) on SDS-PAGE.

Proteins	M (kDa)	log M	R_m
Bovine serum albumin (BSA)	67.0[a]	4.826	0.215
Ovalbumin	43.0[b]	4.634	0.350
α-Chymotrypsinogen	25.7[a]	4.410	0.552
Myoglobin	17.8[a]	4.250	0.741
Cytochrome C	12.4[c]	4.093	0.813
GSCB-I	25.7[d]	-	0.541
GSCB-II	26.6[d]	-	0.561

a : Values taken from Tanford **et al.**, (1968)
b : Value taken from Castellino and Barker (1968)
d : Value taken from Eck and Dayoff (1966)
d : Determined in this study.

The frictional ratio, f/f_0 of both GSCB-I & II obtained from the expression, $f/f_0 = a/(3v_2M/4\P N)^{1/3}$, were 1.22 and 1.28 respectively, where the terms a, v_2, M and N represent the Stokes radius, partial specific volume, molecular weight of the protein and Avogadro's number respectively. The value of partial specific volume, determined from the amino acid composition of human cathepsin B (Ritonja **et al.**, 1985) was 0.742.

ii) Determination of intrinsic viscosity:

The intrinsic viscosity of GSCB-I was determined at 25°C in 60 mM sodium phophate buffer, pH 6.5. The results obtained are shown in Fig 15. The value of intrinsic viscosity, [η], as calculated from the data given in Fig 15, was 3.3 ml/gm.

c) Optical properties :

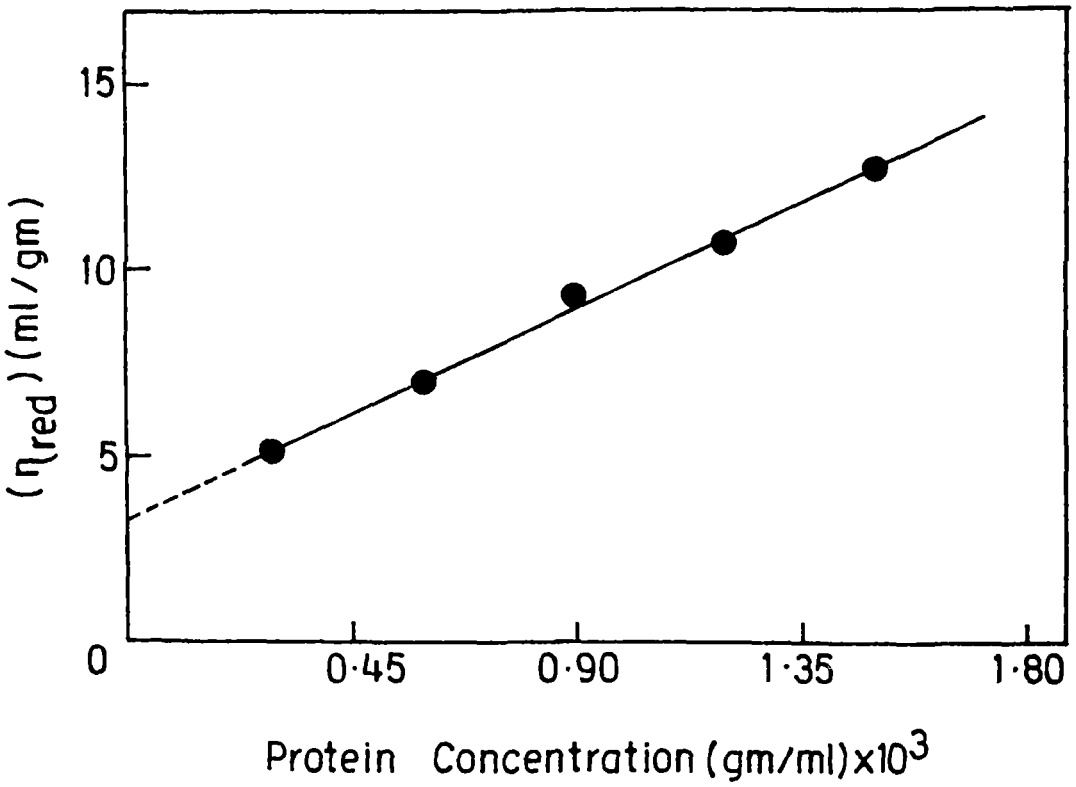
U.V. absorption and fluoroscence spectra of GSCB-I was measured in 60 mM phosphate buffer, pH 6.0, containing 1 mM EDTA and 0.02% sodium azide. The enzyme had a U.V. absorption maxima at 278 nm. The fluoroscence spectra had an excitation near 280 nm and emission maximum near 340 nm. The extinction coefficient ($E^{1\%}_{1cm}$) determined for the enzyme was 15.64.

d) End group analyses :

The NH_2-terminal amino acid residue of GSCB-I was identified using dansylation method. Table V shows the R_f values of standrad amino acids in various solvent systems. The R_f values for the dansyl derivative of the NH_2-terminal amino acid residue of the enzyme was found to correspond with the values determined for the standard dansylated derivative of Leu in the same solvent systems (table VI). These results, therefore, showed that the NH_2 terminal amino acid residue of the enzyme was Leu.

The COOH-terminal amino acid residue of GSCB-I was identified using carboxypeptidase method. The TLC analysis of cathepsin B on

Fig.15 Reduced viscosity of goat spleen cathepsin B-I as a function of protein concentration.
The viscosity was measured in 0.02 M sodium phosphate buffer, pH 6.5 containing 1 mM EDTA at 25°C.


15
10
5
0
(η_{red}) (ml/gm)
0·45
0·90
1·35
1·80
Protein Concentration (gm/ml)x10^3

enzymatic digestion with carboxypeptidase showed only one released amino acid. A comparison of the observed R_f values with those obtained for the standard dansylated amino acids showed that the COOH-terminal amino acid residue of the enzyme was Thr (table VI).

The identified end group amino acids are in accordance with the results reported for cathepsin B from other sources, such as human and rat liver and bovine and buffalo spleen.

e) Determination of free sulfhydryl groups :

The free sulfhydryl contents of GSCB-I under native and denaturing conditions were determined essentially by the method of Ellman. Under native conditions the enzyme was found to contain nearly 0.9 mol of thiol group per mol of protein. In presence of 8 M urea however, the content of thiol group increased to about 1.6 mol per mol of protein. The results thus suggest partial burial of SH-group(s) under native conditions of the enzyme.

f) Determination of total carbohydrate content :

The total carbohydrate content was determined for GSCB-I. The enzyme was found to contain 4.8% carbohydrate as expressed in terms of D-glucose equivalent values obtained from the standard glucose curve. The carbohydrate content reported here though slightly lower, lies within the range of earlier reports on bovine and porcine, liver and spleen sources.

g) Amino acid analysis :

Amino acid composition of GSCB-I was determined using high performance liquid chromatography (HPLC) on a Na^+ column. The results are summarized in table VII. Total tryptophan content of the enzyme was estimated separately using the colorimetric method.

TABLE - V : Relative front (R_f) of dansylated amino acids in thin layer chromatography (TLC) on polymamide sheets.

Dansylated Amino acids	Solvent systems		
	Formic acid (1.5%)	Benzene: Acetic acid (9:1 v/v)	Ethylacetate: Acetic acid:Methanol (20:1:1 v/v)
Alanine	0.574	0.667	0.957
Arginine	-	0.578	0.468
Asparagine	0.596	0.767	0.658
Aspartic acid	0.312	0.233	0.786
Glutamic acid	0.532	0.333	0.689
Glutamine	0.830	0.519	-
Glycine	0.560	0.467	0.956
Isoleucine	0.257	0.953	0.977
Leucine	0.234	0.689	0.844
Lysine	0.816	0.733	0.455
Methionine	0.245	0.756	0.933
Phenylalanine	0.206	0.800	0.933
Proline	0.415	0.933	0.544
Serine	0.768	0 256	0.041
Threonine	0.766	0.222	0.796
Tryptophan	0.128	0.422	0.889
Tyrosine	0.532	0.200	0.828
Valine	0.389	0.711	0.936

TABLE - VI : Identified NH_2- and COOH- terminal amino acid residues of goat spleen cathepsin B (GSCB-I) from their corresponding R_f values on TLC.

Dansylated Amino acid	Solvent systems			Result
	Formic acid (1.5%)	Benzene: Acetic acid (9:1 v/v)	Ethylacetate: Acetic acid: Methanol (20:1:1 v/v)	
NH_2- terminal	0.228	0.688	0.854	Leu
COOH- terminal	0.752	0.222	0.792	Thr

TABLE - VII : Amino acid composition of cathepsin B (GSCB-I) by HPLC.

Amino acid (mol/mol)	Bovine (spleen)[a]	Human (liver)[a]	Porcine (liver)[b]	Rat (liver)[a]	Goat (spleen)[c]
Asx	25	23	25	26	25
Thr	8	13	11	12	9
Ser	21	20	21	21	16
Glx	24	22	24	22	21
Pro	13	15	14	12	15
Gly	33	30	33	33	33
Ala	11	11	14	14	14
Cys	16	14	14	14	ND
Val	14	16	14	14	15
Met	3	4	3	3	5
Ile	14	14	13	15	16
Leu	7	9	11	9	13
Tyr	10	11	11	11	5
Phe	5	8	9	8	9
His	8	8	6	8	6
Lys	11	10	13	8	3
Arg	8	9	10	9	9
Trp	8	8	ND	7	11

a : Baudys **et al.**, (1991)
b : Takahashi **et al.**, (1986)
c : Determined in this study
ND : Not determined

As shown in table VII, the amino acid composition of the enzyme showed close similarities with cathepsin B from other sources such as rat, bovine, human and porcine tissues except for Ser, which was relatively less. Tyr and Lys were significantly lower whereas the amount of Leu and Trp were relatively higher.

C. Effect of physical parameters on the activity and stability of cathepsin B :

a) Effect of pH on activity and stability:

The influence of pH on the activity and stability of GSCB-I was investigated fluorimetrically. The enzyme samples were first exposed to various pHs for 20 min. The cathepsin B activity was then assayed using BANA as substrate at the same pH at 37°C by the standard procedure described in the methods section. The results thus obtained are shown in Fig. 16, curve(a). The maximum activity was found at pH 6.8. The activity declined gradually below 6.8 and sharply above pH 7.0.

The stability of the enzyme at different pH values was checked by incubating the enzyme at different pHs for 20 min at 37°C followed by measurement of the residual activity at pH 6.5 in the usual manner. The result is depicted in Fig. 16, curve(b). The enzyme was found to be stable between pH 4.0-7.0, but was highly unstable above pH 7.0.

b) Effect of temperature :

GSCB-I was incubated at varying temperatures ranging from 8°C to 70°C for 20 min, at pH 6.5. The activity was assayed fluorometrically using BANA as substrate at the corresponding temperatures. The maximal activity of the enzyme was observed at 37°C and decreased rapidly on either side of it (Fig. 17, curve(a)).

Thermal stability of the enzyme was studied by incubating appropriate amount of enzyme at varying temperatures and at pH 6.5 for 20 min followed by measurement of residual activity at 37°C. The results showed that the enzyme is fairly stable upto a temperature of 40°C (for

Fig.16 Effect of pH on the activity and stability of the goat spleen cathepsin B-I.

The enzyme (0.186 mg) was assayed at 37°C in 20 mM tris-acetate buffer of different pHs (a) or at pH 6.5 folowing exposure of the enzyme to various pHs for 20 min (b).

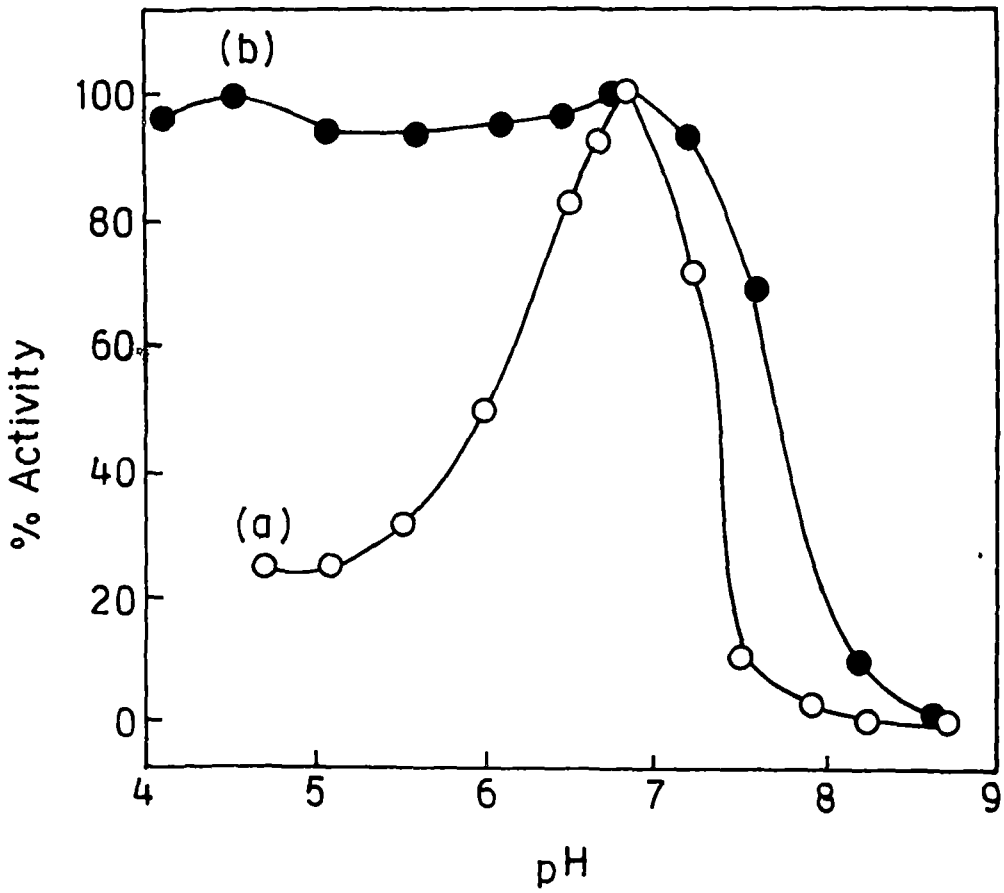
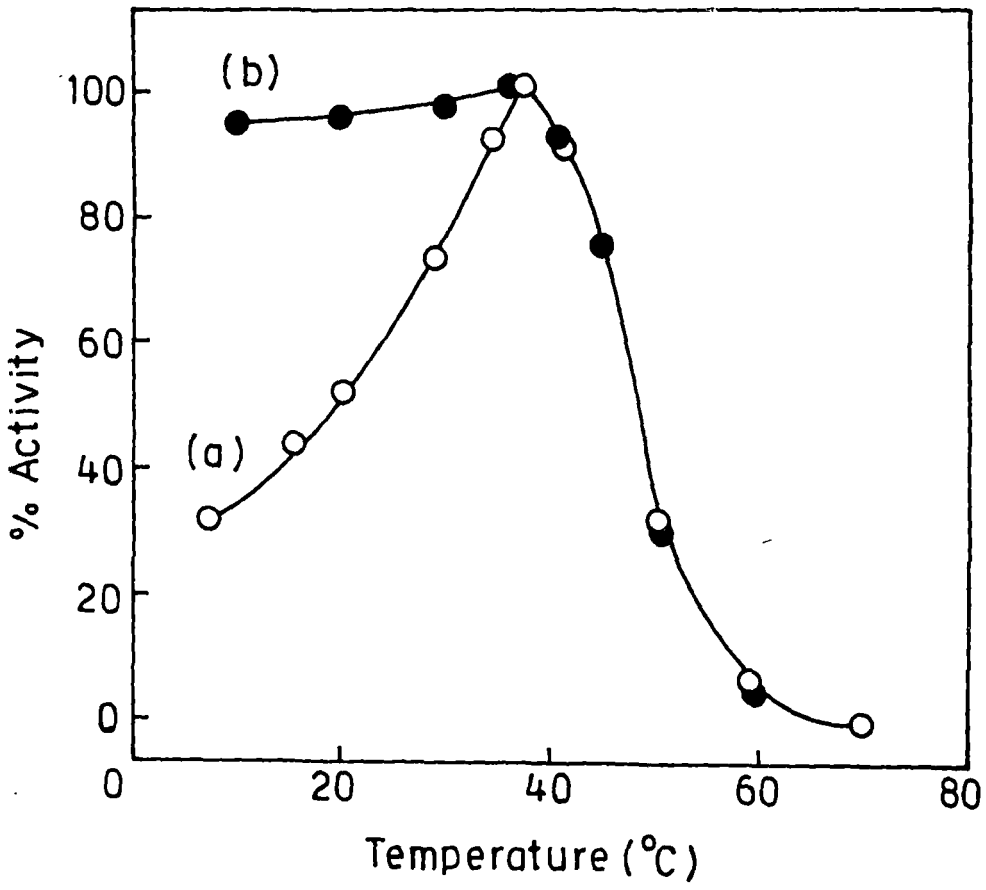

(b)
(a)
100
80
60
40
20
0
% Activity
4
5
6
7
8
9
pH

Fig.17 Effect of temperature on the activity and stability of goat spleen cathepsin B-I. The curves show temperature dependence of the activity (a) and thermal stability (b) of the enzyme. The enzyme (0.096 mg) was assayed in 20 mM tris-acetate buffer, pH 6.5, at different temperatures (a) or at 37°C prior to which the enzyme preparation was exposed to various temperatures for 20 min (b).


(b)
(a)
100
80
60
40
20
0
% Activity
0
20
40
60
80
Temperature (°C)

at least 20 min) but looses its activity rapidly beyond 40°C (Fig. 17, curve(b)).

Our data corroborate that cathepsin B can act as an ideal proteinase in regulating protein turnover at physiological temperature (37°C) and that the catheptic activity can persist for a reasonable time.

c) Effect of ionic strength :

GSCB-I was exposed to sodium phosphate buffer (pH 6.5) of different ionic strengths for an hour prior to enzyme assay at 37°C at the respective ionic strengths. As shown in Fig. 18, a sharp increase in the enzyme activity was observed upto an ionic strength of about 0.022, at which the enzyme activity was maximal. A gradual decrease in activity was seen as the ionic strength was increased above 0.022.

D. Effect of thiol reducing agents:

The effect of various thiol reducing compounds namely L-cysteine, 2 mercaptoethanol, dithiothreitol (DTT), cysteamine, glutathione-SH and thioglycerol on the cathepsin B activity was studied by incubating GSCB-I with varying concentrations of these compounds. The results obtained are shown in Fig. 19. As evident from the results the reducing agents had a very strong stimulatory effect on the enzyme with cysteamine showing highest activation. This verifies that the enzyme from goat spleen is a cysteine proteinase which requires thiol reducing agents for expression of its optimal activity. 2-mercaptoethanol was used in most enzymatic assays because of its higher half-life as compared to others.

E. Effect of thiol blocking agents:

The effects of heavy metal compounds such as $HgCl_2$, $MnCl_2$, thiol blocking agents such as iodoacetic acid and iodoacetamide and ε–amino group modifiers such as maleic anhydride and succinic anhydride have been studied on the activity of GSCB-I, at varying concentrations. The results are summarized in table VIII. As evident from the results, GSCB-I

Fig.18 Effect of ionic strength on goat spleen cathepsin B-I activity. The enzyme (0.056 mg) was assayed at 37°C in sodium phosphate buffer (pH 6.5) of different ionic strengths.

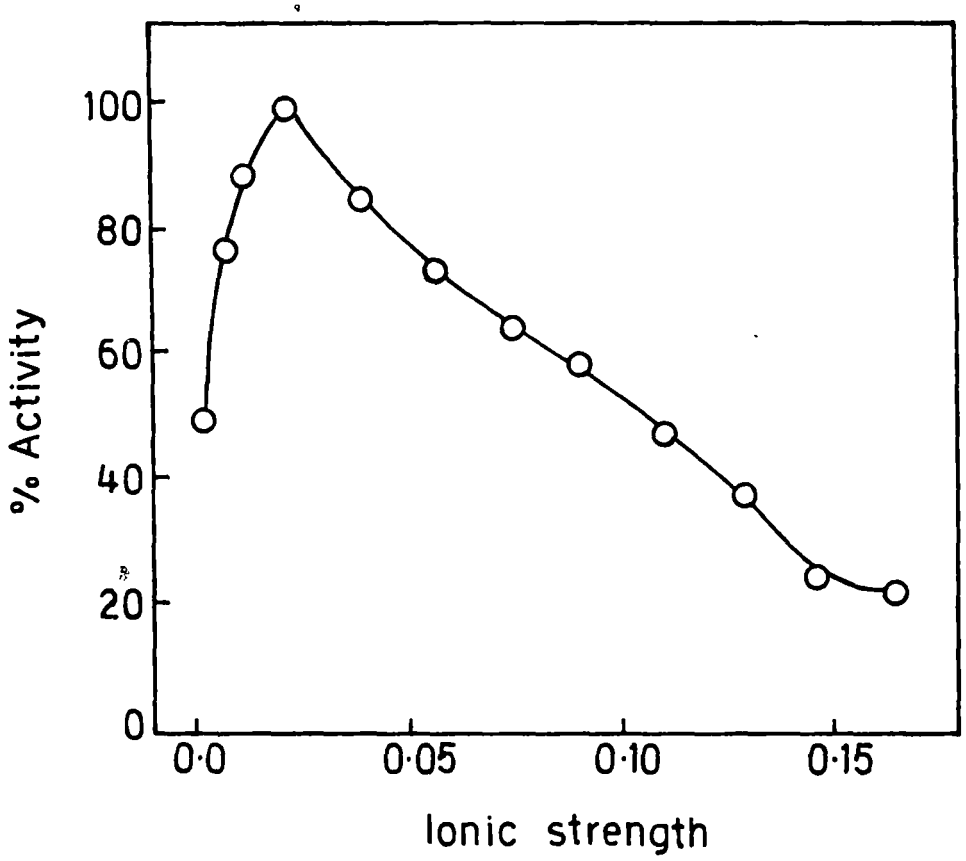
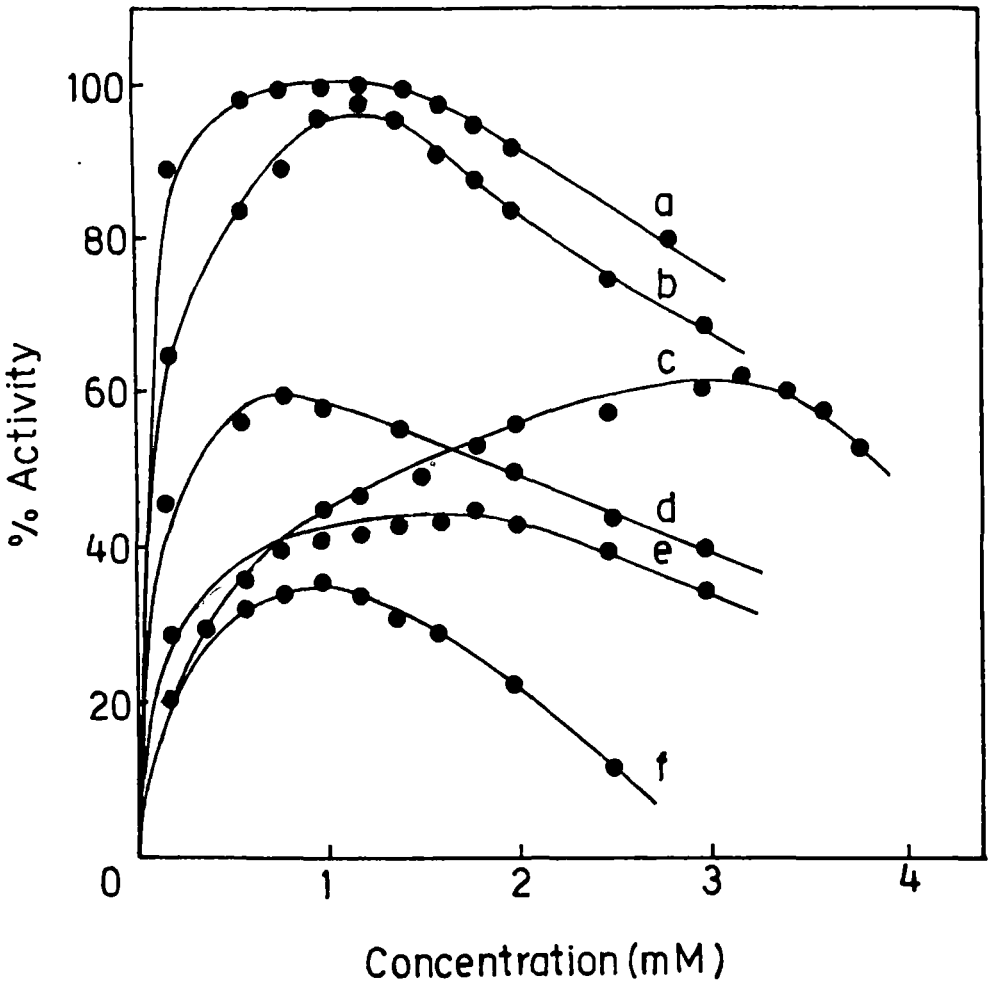

100
80
60
40
20
0
% Activity
0·0
0·05
0·10
0·15
Ionic strength

Fig.19 Effect of various reducing agents on the activity of goat spleen cathepsin B-I.

The enzyme was incubated with (a) Cysteamine; (b) Cysteine; (c) 2-mercaptoethanol; (d) DTT; (e) Glutathione and (f) Thioglyecrol for 30 min and the activity was measured at 37°C in 20 mM sodium phosphate buffer, pH 6.5, using BANA as substrate.


100
80
60
40
20
0
% Activity
1
2
3
4
Concentration (mM)
a
b
c
d
e
f

was found to be very sensitive to heavy metal compounds and thiol blocking agents. However, ε-amino group modifiers had lesser inhibitory effect. This suggests the presence of a thiol group(s) at the active site of the enzyme.

F. Effect of peptidyl inhibitors :

Effect of peptidyl inhibitors, E-64, leupeptin, antipain and pepstatin A on the activity of GSCB-I was studied after exposing the enzyme for 10 min at different inhibitor concentrations. The residual enzyme activity was checked in the usual manner using BANA as the substrate. As shown in table VIII, E-64 proved to be the most potent inhibitor followed by leupeptin and antipain. Pepstatin showed no inhibitory effect. The results thus proved that the enzyme preparation was free from probable contamination with cathepsin D and H since pepstatin inhibits cathepsin D and cathepsin H is less sensitive towards leupeptin.

G. Effect of denaturants :

a) Effect of urea on cathepsin B activity :

Results on inactivation studies of GSCB-I show that the enzyme is very sensitive towards urea (Fig. 20). The enzyme was inhibited by about 50% at a urea concentration of 0.1 M. The reversibility of inactivation of the enzyme was also studied by exposing it at various higher concentrations of urea followed by dilution to lower concentrations of the denaturant. Reversibility of the loss of enzyme activity was possible upto a concentration of 2 M urea. However, exposure of the enzyme to 2.5 M and/or above of the denaturant caused an irreversible loss of enzyme activity.

b) Effect of guanidine hydrochloride (Gdn-HCl) on the activity of cathepsin B :

The effect of Gdn-HCl on the activity of GSCB-I was studied and the results are shown in Fig. 21. Like urea, Gdn-HCl also inactivated the

TABLE - VIII : Effect of various proteinase inhibitors on cathepsin B (GSCB-I) activity*

Additive	Final concentration (mM)	Inhibition (%)
Leupeptin	$1.2x10^{-3}$	100.0
Antipain	$1.5x10^{-3}$	100.0
E-64	$6.0x10^{-5}$	100.0
Pepstatin A	0.10	1.9
$HgCl_2$	0.10	81.3
$MnCl_2$	0.10	51.2
TPCK	0.01	53.1
	0.10	100.0
Maleic anhydride	0.10	9.4
	0.40	25.1
Succinic anhydride	0.10	7.3
	0.40	17.9
Indomethacin	0.10	37.7
Iodoacetic acid	0.01	98.2
Iodoacetamide	0.10	95.3
Gdn-HCl	$0.1x10^3$	50.0
	$1.0x10^3$	100.0
Urea	$0.1x10^3$	41.3
	$1.0x10^3$	95.7

* Enzyme (30 mg/ml) was incubated with the desired compounds at 37°C for 30 min prior to assay of its activity. Values are means of three separate experiments.

Fig.20 Effect of urea on the activity of goat spleen cathepsin B-I.
Activity of the enzyme was measured in the presence of varying concentrations of urea (o–o) at 37°C using BANA as substrate, in 20 mM sodium phosphate buffer, pH 6.5, containing 2 mM each of EDTA and 2-mercaptoethanol. (●) symbols represent regaining of activity after exposure of the enzyme to 1.2 M urea (●–●), 1.5 M urea (●---●), 2.0 M urea (●-·-·●) and at or above 2.5 M urea (●····●) overnight . The activity was regained by diluting the denaturant to lower concentrations. Enzyme activity in the absence of urea was taken as 100%. The concentration of enzyme in each case was 4 µg/ ml.

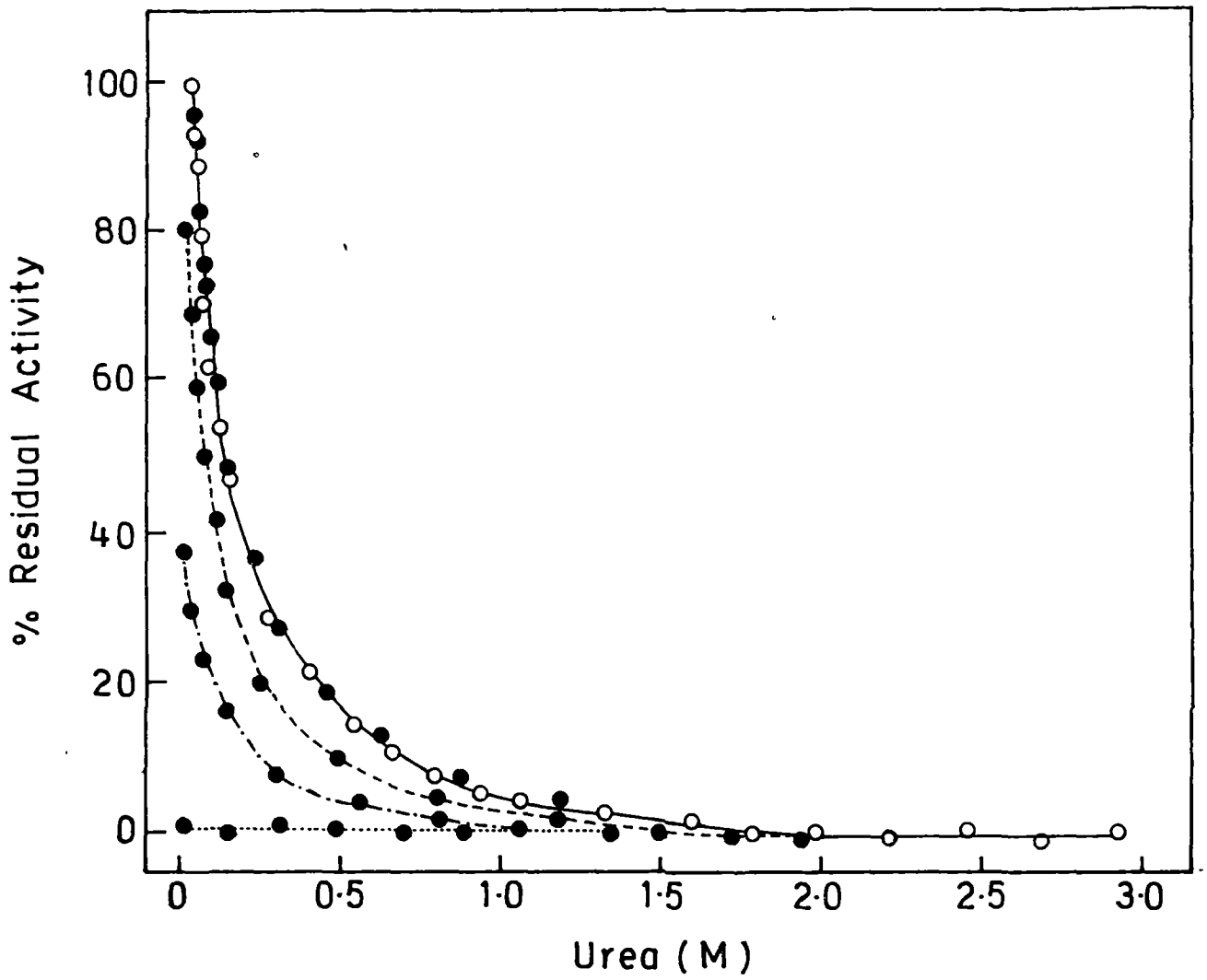
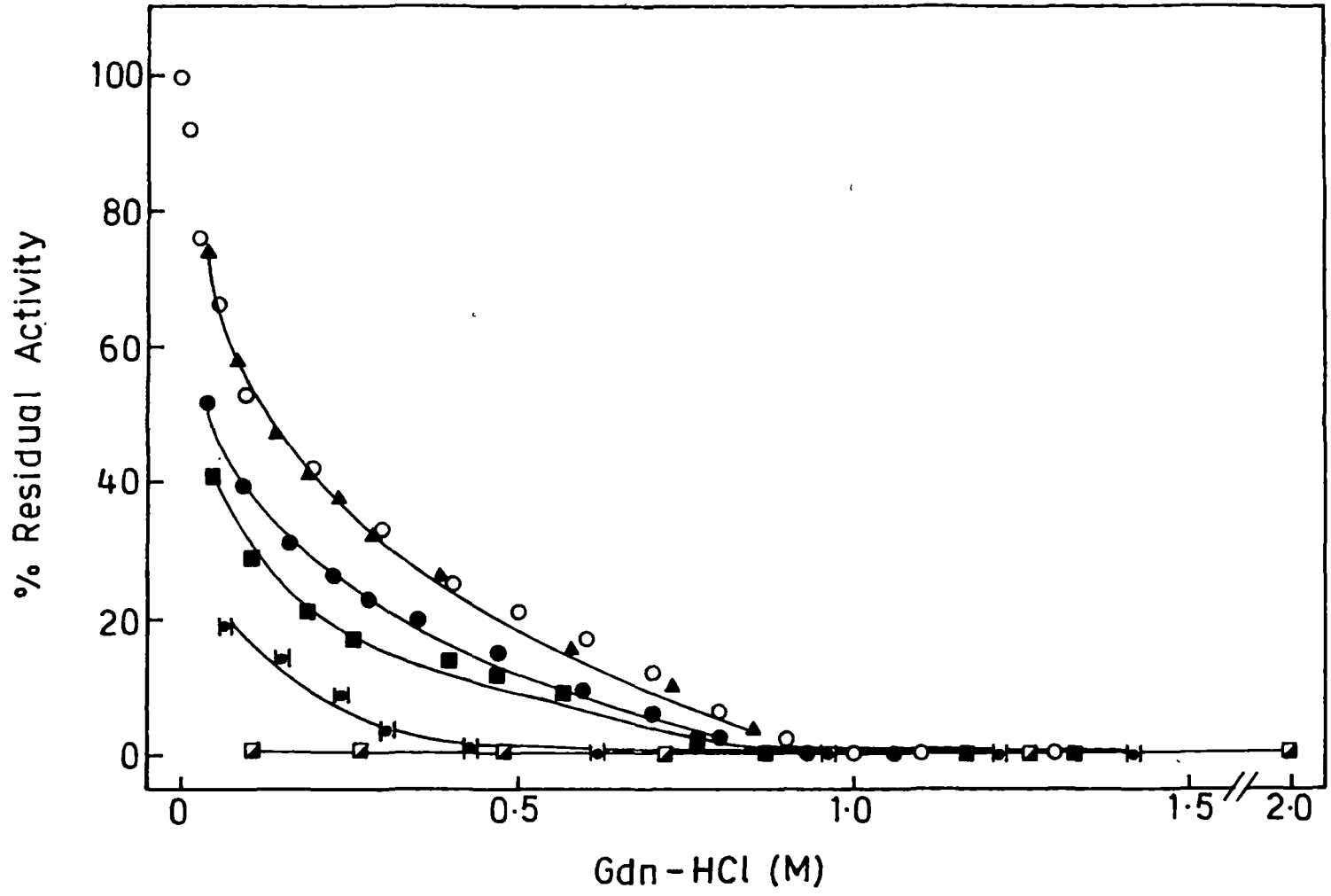

% Residual Activity
100
80
60
40
20
0
0
0·5
1·0
1·5
2·0
2·5
3·0
Urea (M)

Fig.21 Effect of Gdn-HCl on the activity of goat spleen cathepsin B-I. The activity of the enzyme was measured at varying Gdn-HCl concentration (○) at 37°C in 20 mM sodium phosphate buffer, pH 6.5, containing 2 mM each of EDTA and 2-mercaptoethanol. For reactivation studies the enzyme was exposed to 1 M Gdn-HCl (▲), 1.2 M Gdn-HCl (●), 1.4 M Gdn-HCl (■), 1.8 M Gdn-HCl (⋈) and 2.0 M Gdn-HCl (◪) overnight. The residual enzyme activity was measured after diluting the denaturant to lower concentrations. Enzyme activity in absence of Gdn-HCl was taken as 100%. The concentration of enzyme in each case was 4 μg/ml.


% Residual Activity
100
80
60
40
20
0
0
0·5
1·0
1·5
2·0
Gdn-HCl (M)

enzyme. However, the magnitude of inactivation by Gdn-Hcl was higher than urea. The reversibility of the inactivated enzyme was also measured after exposing the enzyme at various Gdn-HCl concentrations and then lowering the denaturant concentrations by dilution. Reversibility of loss of catheptic activity was possible upto to an initial concentration of 1 M Gdn-HCl. However, when the enzyme was exposed to Gdn-HCl concentrations of 2 M and/or above, no activity could be recovered back suggesting an irreversible loss of enzyme activity.

H. Aldolase inactivation by cathepsin B :

Rabbit muscle aldolase was incubated at 37°C with GSCB-I in 0.1 M sodium phosphate buffer, pH 6.5, containing 2 mM 2-mercaptoethanol. Aliquotes of the mixture was taken at various intervals of time during the incubation and the residual aldolase activity was measured according to the method described in the Sigma procedure No. 752. The enzyme showed less affinity for aldolase. About 23% inactivation of aldolase was obtained (Fig. 22) which is much lower than its inhibition by cathepsin B from porcine and bovine sources. Aldolase inactivation by cathepsin B thus shows species and/or tissue specificity.

I. Catalytic properties of cathepsin B :

The values for K_m and V_{max} for different substrates which included synthetic peptides as well as natural protein substrates were computed from the Lineweaver-Burk plot. The results on the kinetic studies of GSCB-I are summarized in table IX. The K_m of the enzyme for the substrates studied followed the order Z-Phe-Arg-MCA < Z-Arg-Arg-MCA < BAPNA < BANA, with the values of 0.07 mM, 0.22 mM, 0.70 mM and 2.64 mM, respectively. The K_m values of GSCB-I fall well within the range for cathepsins from other sources, however the enzyme varies drastically in terms of V_{max}.

Cathepsin B has been shown to be capable of degrading soluble and insoluble collagen, oxidised B-chain of insulin, denatured hemoglobin

Fig.22 Aldolase inactivation by goat spleen cathepsin B-I.
Aldolase was incubated without (A) and with (B) goat spleen cathepsin B-I in a molar ratio of 50:1, in 0.1 M sodium phosphate buffer, pH 6.5, containing 2 mM 2-mercaptoethanol. Residual aldolase activity was measured by taking aliquots at different time intervals.

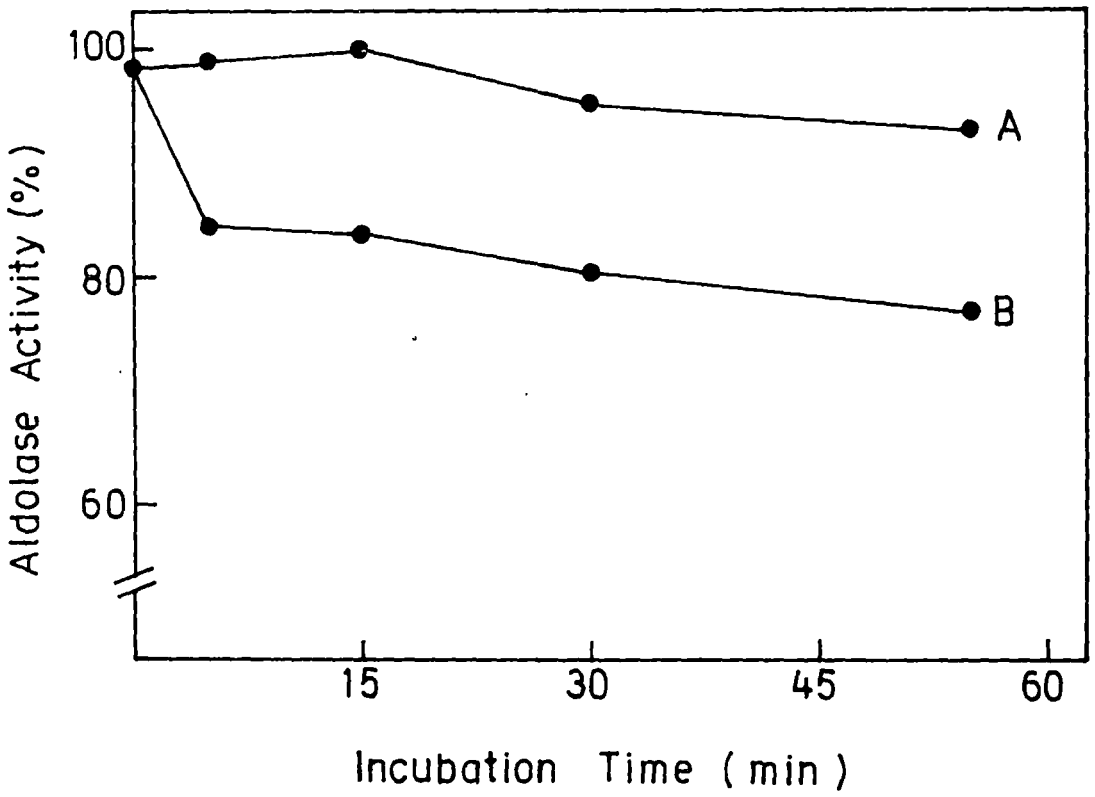

100
80
60
Aldolase Activity (%)
A
B
15
30
45
60
Incubation Time (min)

Fig.23. Lineweaver-Burk plot of GSCB-I with BAPNA as substrate. All the enzymatic activity measurements were performed at 37°C in 20 mM sodium phosphate buffer, pH 6.5, containing 2 mM each of EDTA and 2-mercaptoethanol. The bar represents the standard deviation (SD), values of three independent experiments.

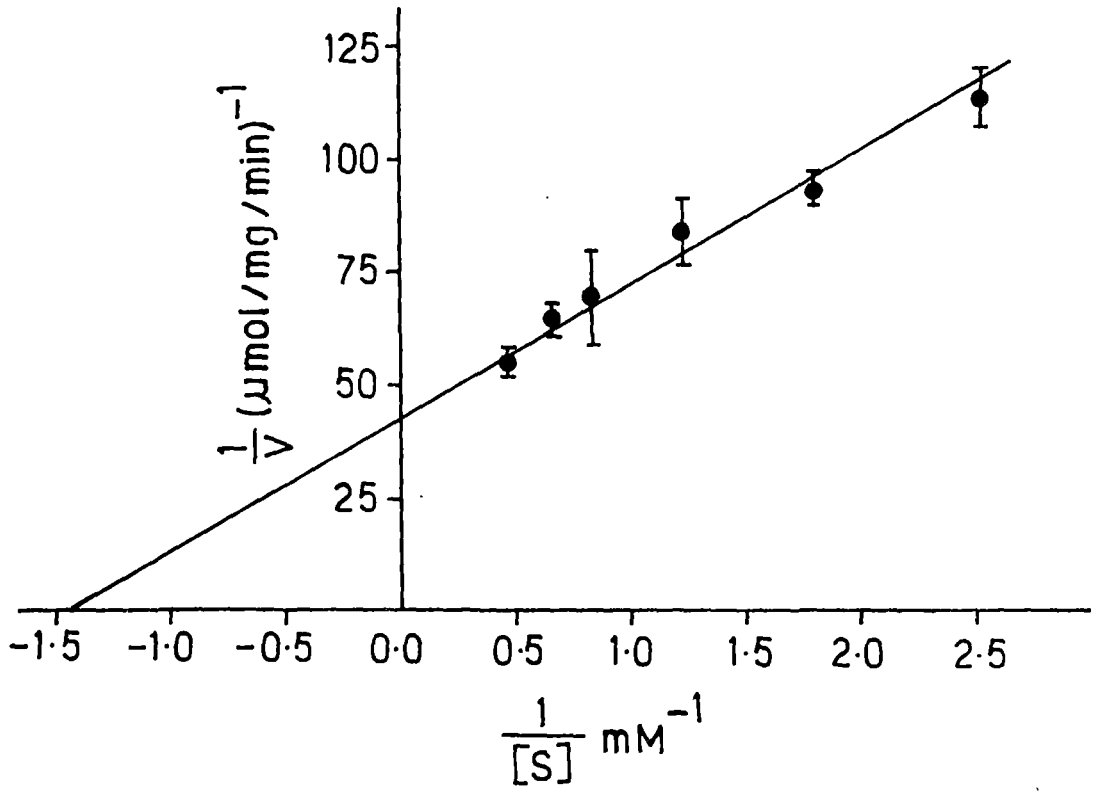
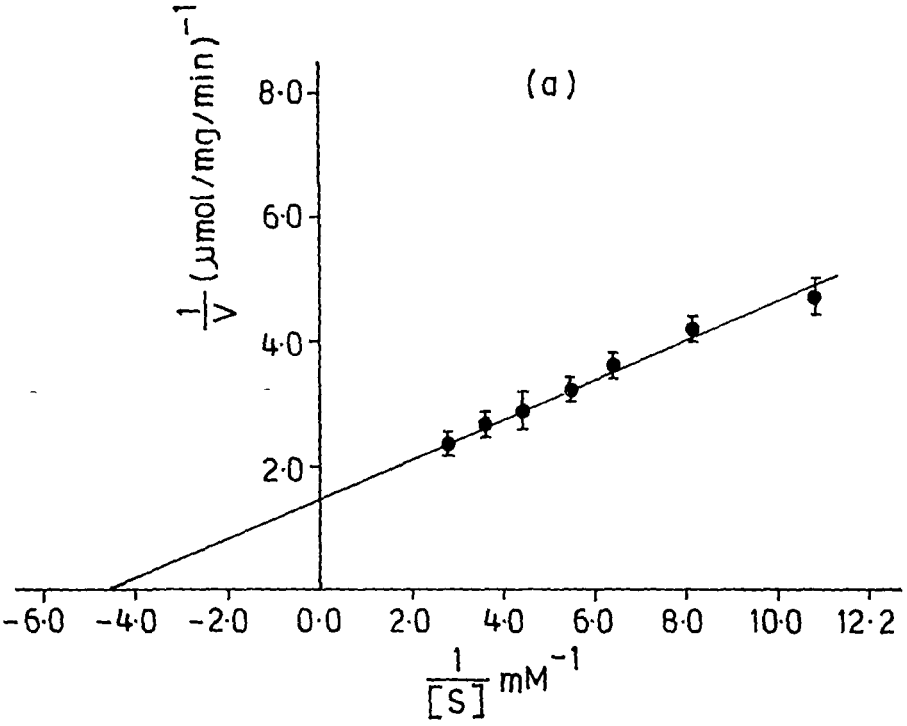

125
100
75
50
25
$\frac{1}{v}$ (μmol/mg/min)$^{-1}$
-1·5
-1·0
-0·5
0·0
0·5
1·0
1·5
2·0
2·5
$\frac{1}{[S]}$ mM^{-1}

Fig.24 Lineweaver-Burk plots of GSCB-I with Z-Arg-Arg-MCA (a) and Z-Phe-Arg-MCA (b), as substrates.
All the enzymatic activity measurements were performed at 37°C in 340 mM sodium acetate buffer, pH 6.5, containing 4mM EDTA and 8mM DTT. The bar represents the SD of values of three independent experiments.


(a)
$\frac{1}{V}$ (μmol/mg/min)$^{-1}$
8·0
6·0
4·0
2·0
-6·0
-4·0
-2·0
0·0
2·0
4·0
6·0
8·0
10·0
12·2
$\frac{1}{[S]}$ mM^{-1}

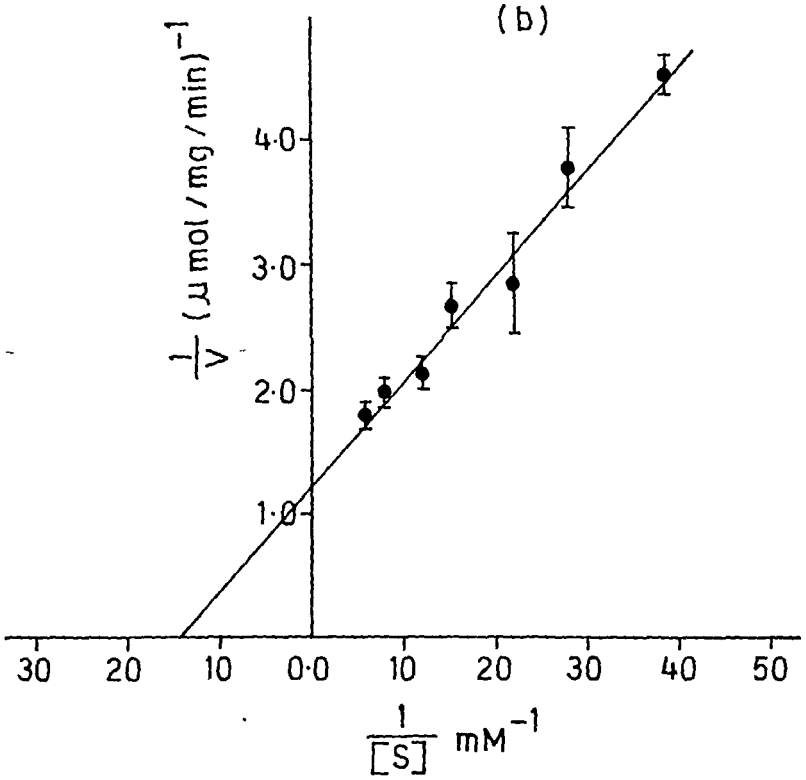
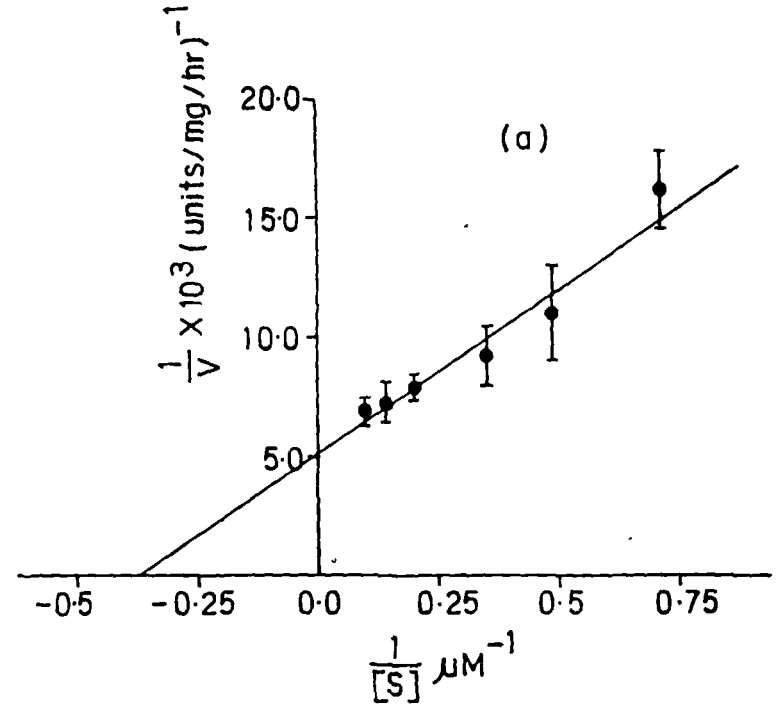

(b)
$\frac{1}{V}$ (μ mol / mg / min)$^{-1}$
4·0
3·0
2·0
1·0
30
20
10
0·0
10
20
30
40
50
$\frac{1}{[S]}$ mM^{-1}

Fig.25 Lineweaver-Burk plots of GSCB-I using BSA (a) and bovine milk casein (b), as substrates.
The experimental conditions were similar to those described in legend to Fig.23.


(a)
$\frac{1}{V} \times 10^3$ (units/mg/hr)$^{-1}$
20·0
15·0
10·0
5·0
-0·5
-0·25
0·0
0·25
0·5
0·75
$\frac{1}{[S]}$ µM^{-1}

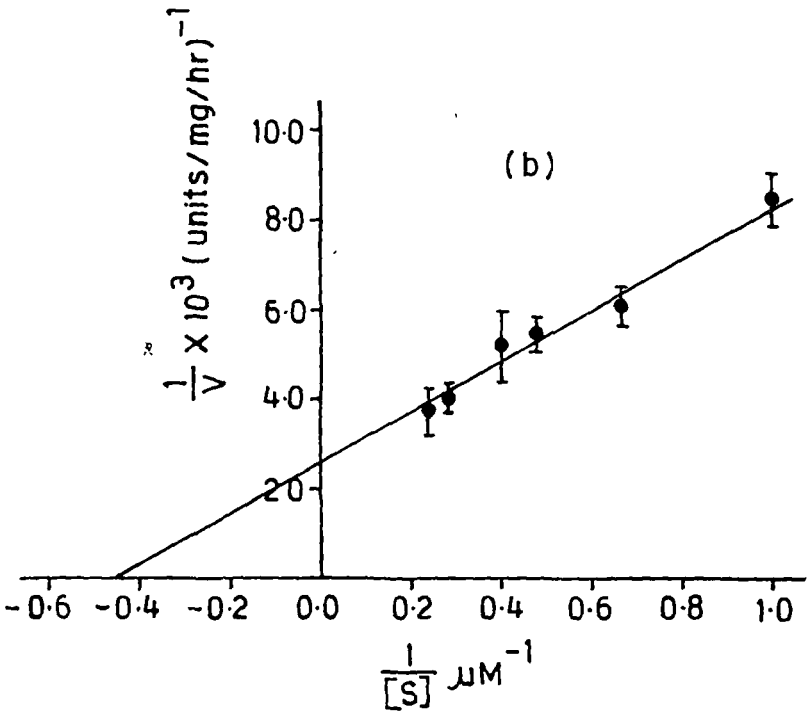

(b)
$\frac{1}{V} \times 10^3$ (units/mg/hr)$^{-1}$
10·0
8·0
6·0
4·0
2·0
-0·6
-0·4
-0·2
0·0
0·2
0·4
0·6
0·8
1·0
$\frac{1}{[S]}$ µM^{-1}

Fig.26 Lineweaver-Burk plot of GSCB-I using goat hemoglobin as substrate.
The experimental conditions were similar to those described in legend to Fig.23.

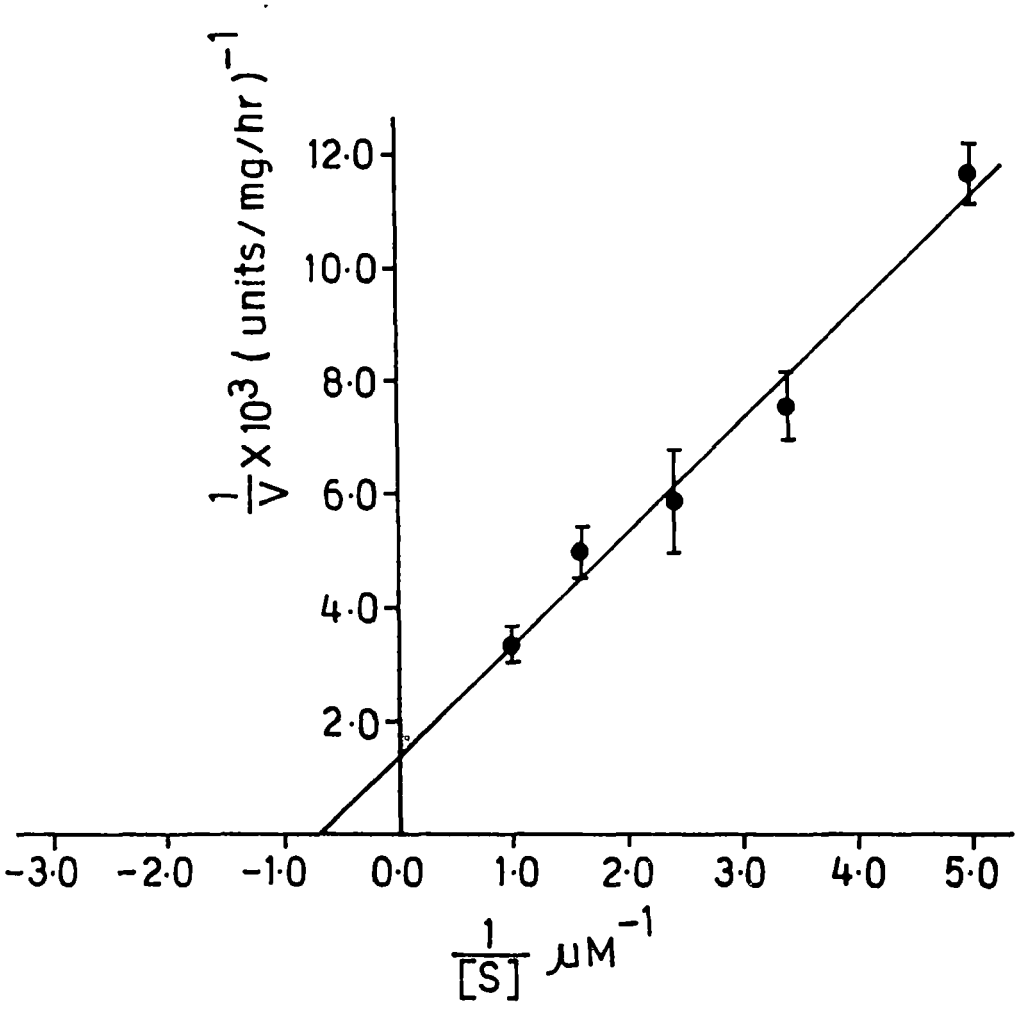

$\frac{1}{V} \times 10^3$ (units / mg/hr)$^{-1}$
12·0
10·0
8·0
6·0
4·0
2·0
-3·0
-2·0
-1·0
0·0
1·0
2·0
3·0
4·0
5·0
$\frac{1}{[S]}$ μM^{-1}

TABLE - IX : Kinetic parameters of cathepsin B (GSCB-I) from goat spleen.

Substrate	K_m	V_{max}
BANA	2.64 mM	0.51 units[a]
BAPNA	0.70 mM	0.03 "
Z-Phe-Arg-MCA	0.07 mM	0.81 "
Z-Arg-Arg-MCA	0.22 mM	0.67 "
BSA	2.67 μM	0.20×10^3 units[b]
Casein	2.18 μM	0.38×10^3 "
Hemoglobin	1.46 μM	0.67×10^3 "

[a] One unit represents the amount of enzyme required to release 1 μmol of product per min.

[b] One unit corresponds to the amount of enzyme required to increase the colour produced by 0.01 O.D. unit per hour under our assay conditions.

and human fibrinogen at acidic pH. GSCB-I has been found to be very effective against denatured hemoglobin, casein and BSA. Goat hemoglobin proved to be the most effective substrate having a K_m value of 1.46 µM followed by casein with a K_m value of 2.18 µM and BSA with a K_m value of 2.67 µM.

J. Immunological studies :

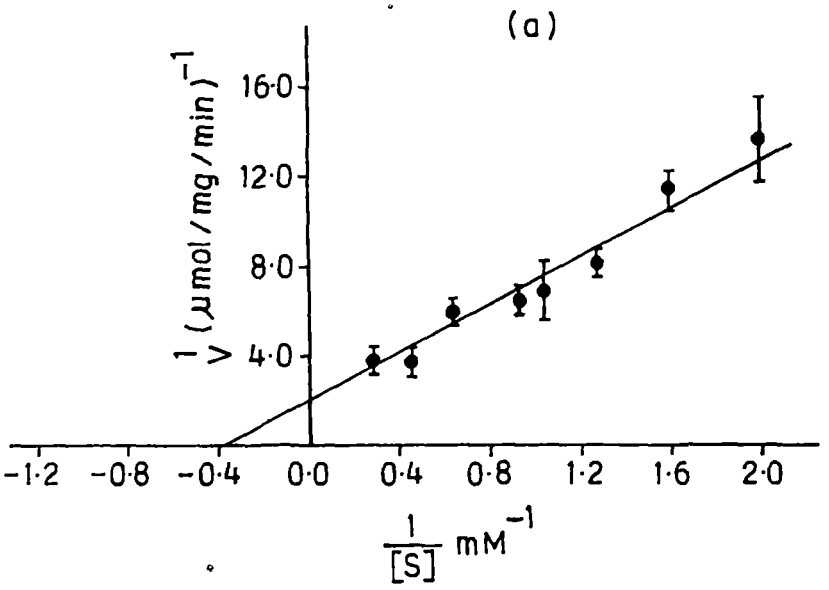
Polyclonal antibodies against unresolved goat spleen cathepsin B were raised in rabbits. These antibodies cross-reacted specifically with the purified goat spleen cathepsin B (unresolved or GSCB-*I* and *II*) and not with cathepsin H, a homologous cysteine proteinase from either buffalo kidney or porcine lung (Fig. 29 & 30). Similarly, no precipitin line was detected even when buffalo kidney cathepsin B was used as an antigen (Fig. 29).

K. Comparative study of GSCB-I & II :

In addition to determination of molecular weights of GSCB-I & II by gel filtration and SDS-PAGE, effects of various inhibitors on them were also studied. Both the enzyme fractions had similar sensitivity towards urea, iodoacetamide, antipain and leupeptin inactivation. However, slight differences in their inhibition with iodoacetic acid could be seen (table X). Kinetic studies of both the enzyme preparations were performed using substrates such as BANA, Z-Arg-Arg-MCA and Z-Phe-Arg-MCA. The results summarized in table XI, clearly indicate that both of them catalyze similar substrates but differ in their kinetic properties.

Thus from the results obtained, GSCB-I & II are probably isozymes of cathepsin B from goat spleen.

Fig.27 Lineweaver-Burk plots of GSCB-I (a) and GSCB-II (b) with BANA as substrate.
The experimental conditions were similar to those described in legend to Fig. 23.


(a)
16·0
12·0
8·0
4·0
$\frac{1}{V}$ (μmol/mg/min)$^{-1}$
-1·2
-0·8
-0·4
0·0
0·4
0·8
1·2
1·6
2·0
$\frac{1}{[S]}$ mM^{-1}

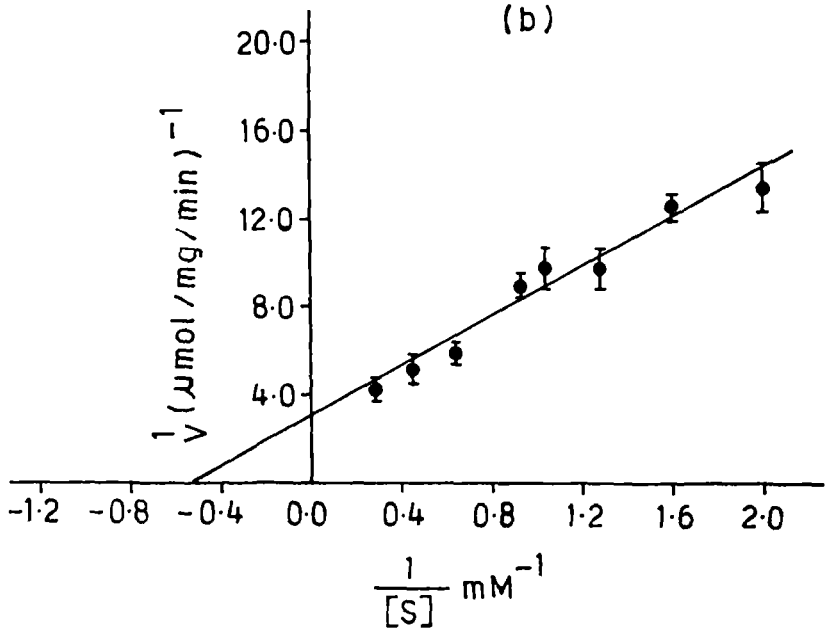
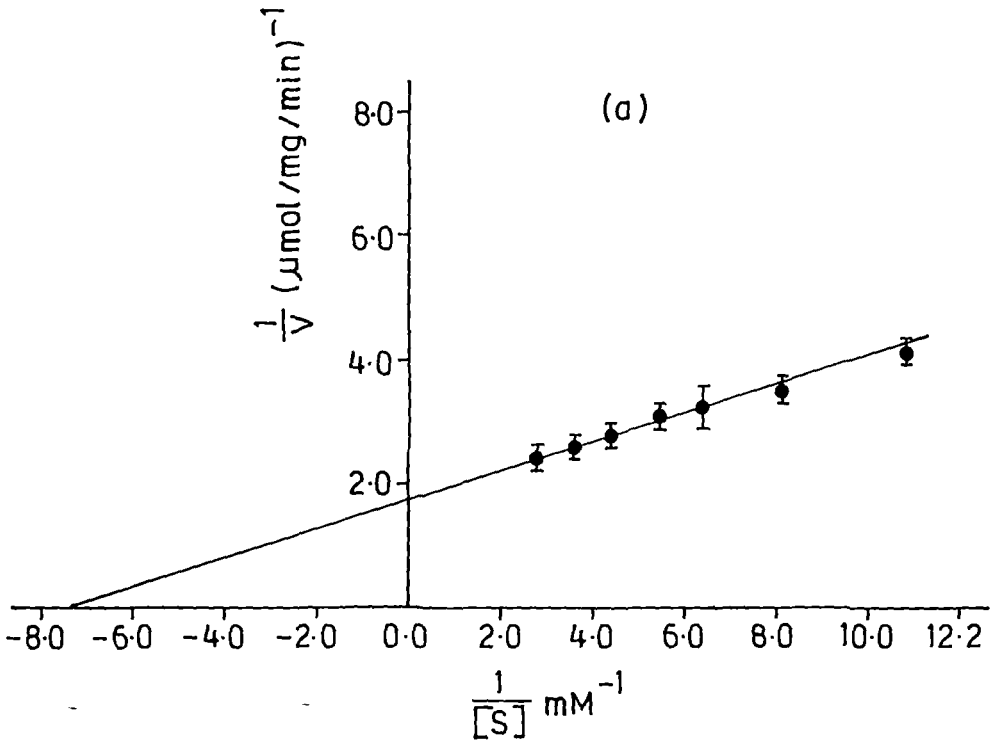

(b)
20·0
16·0
12·0
8·0
4·0
$\frac{1}{V}$ (μmol/mg/min)$^{-1}$
-1·2
-0·8
-0·4
0·0
0·4
0·8
1·2
1·6
2·0
$\frac{1}{[S]}$ mM^{-1}

Fig.28 Lineweaver-Burk plots of GSCB-II with Z-Arg-Arg-MCA (a) and Z-Phe-Arg-MCA (b), as substrates.
The experimental conditions were similar to those described in legend to Fig.24 .


(a)
$\frac{1}{V}$ (μmol/mg/min)$^{-1}$
8·0
6·0
4·0
2·0
-8·0 -6·0 -4·0 -2·0 0·0 2·0 4·0 6·0 8·0 10·0 12·2
$\frac{1}{[S]}$ mM^{-1}

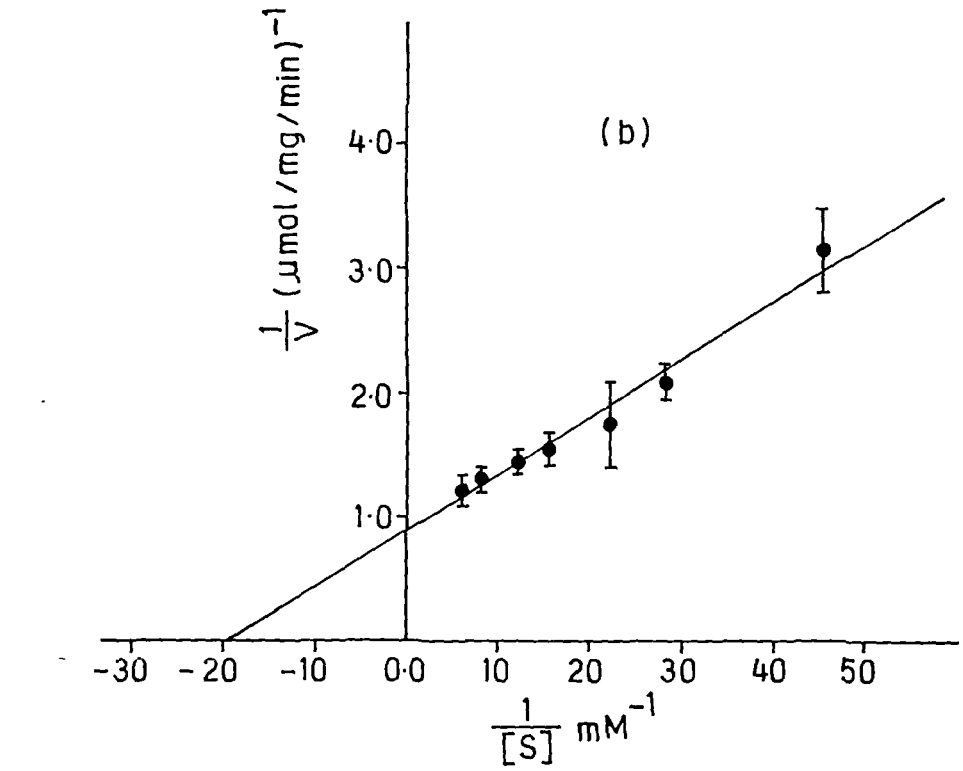

(b)
$\frac{1}{V}$ (μmol/mg/min)$^{-1}$
4·0
3·0
2·0
1·0
-30 -20 -10 0·0 10 20 30 40 50
$\frac{1}{[S]}$ mM^{-1}

TABLE - X : Comparison of molecular properties of goat spleen cathepsin B isozymes (GSCB-I and GSCB-II).

	GSCB -I	GSCB -II
Molecular weight (kDa)		
SDS-PAGE	25.7	26.6
Gel filtration	28.1	31.6
Binding to ConA-Sepharose 4B	No	Yes
Specific activity (units/mg protein) for Z-Arg-Arg-MCA	4.16	1.06
Pepstatin inhibition	No	No
Leupeptin inhibition	Yes	Yes
Antipain inhibition	Yes	Yes
Inhibition with thiol blocking reagents		
i) Iodoacetic acid (0.54 μM)	30%	40%
ii) Iodoacetamide (0.54 MM)	03%	04%

TABLE - XI : Kinetic properties of goat spleen cathepsin B isozymes.

Substrate	GSCB-I		GSCB-II	
	K_m (mM)	V_{max} (units)*	K_m (mM)	V_{max} (units)*
BANA	2.64	0.51	1.87	0.32
Z-Arg-Arg-MCA	0.22	0.67	0.14	0.58
Z-Phe-Arg-MCA	0.07	0.81	0.05	1.11

* One unit of enzyme is defined as the amount of enzyme required to release 1 μmol of product per min.

Fig.29 Ouchterlony double immunodiffusion of anti-goat spleen cathepsin B antiserum against cathepsins B and H.
About 150 µl of antiserum was taken in the central well and 60 µl of purified (a) cathepsin B from goat spleen; (b) cathepsin B from buffalo kidney; (c) cathepsin H from buffalo kidney and (d) cathepsin H from porcine lung was applied in the specified wells and incubated overnight at 37°C in phosphate buffered saline, at pH 7.2.

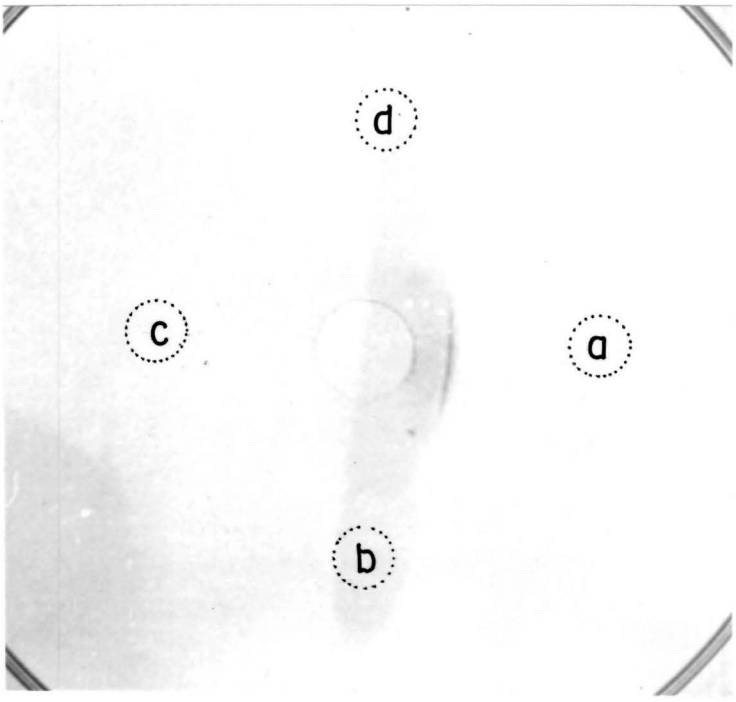
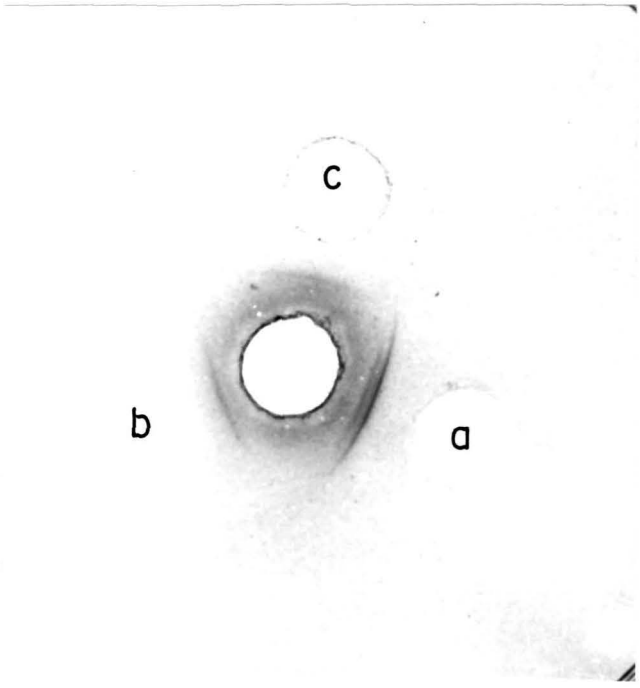

d
c
a
b

Fig.30 Ouchterlony double immunodiffusion of goat spleen anti-cathepsin B antibody with unresolved goat spleen cathepsin B,goat spleen cathepsin B-I and II.

About 150 µl of antiserum was loaded in the central well and 60 µl of GSCB-I (b), GSCB-II (c) and unresolved goat spleen cathepsin B (a) were applied in the specified wells and incubated overnight at 37°C in phosphate buffered saline, at pH 7.2.


c
b
a

DISCUSSION

Cathepsins have drawn considerable interest due to their possible involvement in various physiological and pathological processes (Sano **et al.**, 1993; Laszlo **et al.**, 1993; Trabandt **et al.**, 1991; Kenessey **et al.**, 1989; Mizuno **et al.**, 1982). The outcome of research on cathepsin B. particularly on its isozymes from different laboratories, have not been unequivocal. While the enzyme from some tissues was found to be homogeneous on polyacrylamide gels, multiplicity observed in protein bands of cathepsin B preparations from other sources have been attributed to microheterogeneity representing some related proteins and/or degradation products of the active enzyme (Muno **et al.**, 1990; Ahmad **et al.**, 1989; Agarwal & Khan, 1987b; Fazili & Qasim, 1986; Takahashi **et al.**, 1984 a; Barrett & Kirschke, 1981).

The purification procedure described herein included some changes as against the earlier method of purification of cathepsin B (Ahmad **et al.**, 1989). Cathepsin H could be easily separated from the enzyme preparation by the use of DEAE-Cellulose (Fig. 3). The use of CM-Sephadex chromatography gave a single peak at pH 5.6 which showed high BANA-lyase activity characteristic of cathepsin B (Fig. 4). No activity against substrates for cathepsin H could be observed by the enzyme fractions eluting at this peak. Thus use of DEAE-Cellulose before CM-Sephadex chromatography helped in a clear separation of cathepsin B from H. A striking feature of the goat spleen cathepsin B is its elution from the CM-Sephadex ion exchange column at a relatively lower pH and ionic strength. The enzyme was eluted at pH 5.6 as against pH 6.0 and 0.1 M NaCl, required for a similar elution of cathepsin B from porcine spleen (Takahashi **et al.**, 1984a & Fig. 4). This may be attributed to the differences in the nature and extent of glycosylation (Takahashi **et al.**, 1984a, 1986a) and/or amino acid composition of the enzyme from the two sources.

Cathepsin B from goat spleen after CM-Sephadex chromatography step, yielded a major and a minor protein band on PAGE, which could

however be resolved by affinity chromatography on a ConA-Sepharose 4B column. The fraction which did not bind to ConA-Sepharose was designated as goat spleen cathepsin B-I (GSCB-I) and the fraction which eluted with methylglucose was designated as goat spleen cathepsin B-II (GSCB-II) (Fig. 5). The differential binding of cathepsin B to ConA-Sepharose indicated that cathepsin B from goat spleen is heterogeneous in carbohydrate structures and GSCB-I contained carbohydrates differing from the high mannose-type oligosaccharides usually found in lysosomal hydrolases (Takahashi **et al.**, 1983; Natowicz **et al.**, 1982). The molecular weights determined by SDS-PAGE for GBSC-I & II were found to be 25.7 kDa and 26.6 kDa, respectively. The molecular weight of GSCB-I & II determined by analytical gel filtration yielded a value of 28.1 kDa and 31.6 kDa respectively. The molecular weights determined both by analytical gel filtration and SDS-PAGE are in agreement with the earlier reported values of 23-29 kDa for cathepsin B from other sources (Ahmad **et al.**, 1989; Agarwal & Khan.,' 1987b; Takahashi **et al.**, 1986a). The molecular weight as determined by gel filtration was found to be comparatively higher than that determined by SDS-PAGE. Since the enzymes are glycoproteins and therefore have an enhanced tendency for hydration (Tanford, 1961), molecular weight could be over estimated by gel filtration.

A comparative study of GSCB-I & II showed that both the enzyme fractions had similar sensitivities towards urea, antipain, leupeptin, iodoacetic acid and iodoacetamide inhibition (table X). The inactivation of GSCB-I & II by more than 50% at a urea concentration of about 1.0 M and absence of inhibition by pepstatin ruled out the possible contamination by cathepsin L (Barrett **et al.**, 1981) and cathepsin D (Nishimura **et al.**, 1988) respectively, as cathepsin L activity is retained even above 3.0 M urea and pepstatin is a very potent inhibitor of cathepsin D. Also absence of activity against Leu-NA, Arg-NA and Arg-MCA ruled out the presence of leucine aminopeptidase and/or cathepsin H from our enzyme preparations (Bromme **et al.**, 1993; Baudys **et al.**, 1991; Kirschke **et al.**, 1983). Antibodies raised against unresolved goat spleen cathepsin B cross-reacted distinctly with both the enzyme fractions (GSCB-I & II) in Ouchterlony double immunodiffusion (Fig. 30), which indicated that the native

unresolved cathepsin B from goat spleen shares the antigenic determinants present in both the fractions. It also entails that antibodies can be raised against the native goat spleen cathepsin B and that it does not undergo rapid denaturation following immunization as against earlier reports on cathepsin B from other sources (Barrett, 1973; Mort **et al.**, 1980).

Differences in the kinetic parameters of GSCB-I & II showed that they have the same substrate requirements but differed considerably in their kinetic properties (table XI). It was thus assumed that these two fractions could represent two isozymes and their presence in the goat spleen can be accounted for, since spleen is histologically a complex organ having different cell types with varying degrees of differentiation, with enzyme species having different maturational states.

However, as already stated in the results section, most of the experiments in this study were done with GSCB-I because of its higher specific activity and yield compared to GSCB-II.

The hydrodynamic properties studied on a calibrated Sephadex G-75 column showed a close resemblance of the properties of GSCB-I such as molecular weight (28.1 kDa), Stokes radius (2.46) and frictional ratio (1.22) with that of the reported values of buffalo liver cathepsin B (Fazili & Qasim, 1986) suggesting that the goat enzyme is similar to the buffalo liver enzyme. However, it differed in the values of molecular weight, Stokes radius and frictional ratio from buffalo spleen cathepsin B (Ahmad **et al.**, 1989) clearly suggesting a tissue/species dependence of the enzyme.

The results on intrinsic viscosity of GSCB-I (Fig. 15) was found to be 3.30 ml/gm, which is well within the range (3.0-4.0 ml/gm) expected for native proteins having compact and globular conformations (Tanford, 1968).

The results on end group analysis showed Leu to be the NH_2- and Thr, the COOH- terminal amino acid residues, which were found to be the same as in cathepsin B from other sources (Ahmad & Khan, 1990; Meloun **et al.**, 1988; Takahashi **et al.**, 1986a, 1984a; Takio **et al.**, 1983).

A total of 0.9 and 1.6 moles of thiol groups could be titrated per mole of the protein in the absence and presence of 8 M urea, respectively. These results are in agreement with the values determined for cathepsin B from other sources (Sumiya **et al.**, 1992; Musil **et al.**, 1991; Baudys **et al.**, 1990; Otto, 1971). The results clearly showed that the thiol group(s) in the enzyme is/are not fully exposed.

The amino acid composition of the enzyme showed close similarities with cathepsin B from rat, bovine, human and porcine sources, except for Ser, Tyr and Lys which were significantly lower in goat spleen cathepsin B. In contrast Leu and Trp were present in relatively higher amounts (table VII).

U.V. absorption spectra of the enzyme had a maxima at 278 nm. The specific extinction coefficient, $E^{1\%}_{1cm}$ of the enzyme was determined to be 15.64. This agrees with the value (15.50) for bovine spleen cathepsin B (Bajkowski & Frankfater, 1983). However, it is certainly higher (15%) from the value (13.2) obtained for buffalo spleen cathepsin B (Ahmad & Khan, 1990), suggesting an increased number of aromatic chromophores particularly Trp in goat or bovine cathepsin B than the buffalo enzyme. The fluorescence spectra (excitation near 280 nm and emission maximum near 340 nm) were again the characteristic of proteins containing Trp residue (Teale, 1960).

The isoionic pH of GSCB-I was found to be 5.12 which is consistent with the values of 4.8-5.3 for the enzyme from different sources (Tanaka **et al.**, 1984; Barrett **et al.**, 1981) indicating an acidic nature of the enzyme.

In addition to the reducing agent, 2-mercaptoethanol, which serves as an activator for cathepsin B in the usual assay because of its higher half-life as compared to others (Agarwal & Khan., 1987a; Evans **et al.**, 1983), the activation of GSCB-I was also studied by the other thiol reducing compounds. The results thus obtained are depicted in Fig. 19. All the reducing agents showed very strong stimulatory effect on enzyme activity. Among the thiol modulators tested, cysteamine was most effective

and thioglycerol was least effective. These results indicate that the goat spleen enzyme is a cysteine proteinase requiring reduced thiol groups for its activity.

Influence of different proteinase inhibitors on GSCB-I activity summarized in table VIII, shows divalent cations such as Mn^{++} and Hg^{++}, alkylating agents like iodoacetic acid and iodoacetamide and peptidyl inhibitors, E-64, antipain and leupeptin inactivated the enzyme. These results suggested the involvement of cysteine group(s)in the expression of catalytic activity of the enzyme. Maleic and succinic anhydride used for the modification of α amino acid residue of the proteins, showed very mild inhibitory effect on the activity of cathepsin B, suggesting that, probably such modifications of lysyl ε-amino groups of the enzyme do not have much effect on the active site conformation.

An integral part of inflammatory response in tissue destruction is by lysosomal proteolytic enzymes and therefore, inhibitors of these could be used as therapeutic agents. As pathways of tissue breakdown by proteolytic attack and prostaglandin biosynthesis represent the major inflammatory responses, dual inhibitors of these pathways may have an advantage in treatment of inflammatory diseases. Indomethacin, a non steroidal drug, is known to inhibit prostaglandin biosynthesis. In this study, indomethacin (0.1 mM) caused about 38% inhibition of the enzyme activity. This agrees well with the data of 37% inhibition of rat spleen cathepsin B. Inhibition of cathespin B activity by indomethacin has been attributed to a change brought about in the conformation of the enzyme (Yamamoto **et al.**, 1984). This drug might help reduce inflammation by inhibiting cathepsin B activity and lowering the rate of proteolysis.

The activity of GSCB-I was markedly inhibited by urea and Gdn-HCl (Fig. 20 & 21). About 50% inactivation of the enzyme was achieved at a urea concentration of 0.15 M and virtually no activity could be observed above 1.0 M. The enzyme got irreversibly inactivated as the urea concentration was raised beyond 2.5 M. Gdn-HCl was found to be more effective and 50% inactivation was achieved at 0.12 M. The loss of enzyme activity was reversible at and below 1.8 M Gdn-HCl, but above

2.0 M concentration of the denaturant the enzyme irreversibly lost its activity. The irreversible loss of catheptic activity at 2.5 M and 2.0 M concentrations of urea and Gdn-HCl respectively is possibly because of some perturbations near the active site of the enzyme and probably not because of loss of three dimensional structure, as low concentrations of denaturants may not be enough to bring about a conformational change (Khan **et al.**, 1992; Agarwal & Khan., 1988). TPCK showed a moderate inhibitory effect on GSCB-I. At 10 μM TPCK, the inhibition of the goat enzyme was about 53% and it showed complete inactivation at a TPCK concentration of 100 μM and above. This indicates that cathepsin B activity may be similar to chymotryptic activity in the mode of inhibition by TPCK. Iodoacetic acid was found to be 10 times more effective than iodoacetamide in inhibiting GSCB-I activity (table VIII). The degree of inhibition corresponds with the earlier findings of porcine parathyroid cathepsin B (MacGregor **et al.**, 1979). The reason for the effectiveness of iodoacetic acid may be due to a strong binding between the -ve charge of the COOH- group of iodoacetic acid and the +ve charge at Arg 200 located close to the active site His 199 as found in porcine (Takahashi **et al.**, 1986b), rat (Takio **et al.**, 1983) and human (Ritonja **et al.**, 1985) cathepsin B.

Cathepsin B is generally believed to be active only at acidic pH (Kirschke **et al.**, 1980; McDonald & Ellis, 1975) and therefore unlikely to play a role in protein breakdown under physiological conditions where the pH is maintained between 7.0-7.5 (Mort **et al.**, 1984). GSCB-I was found to have a pH optimum at pH 6.8 which is comparable to the value reported by Fok and Paeste, 1982 but is significantly higher than the values reported by others (Zvonar-Popovic **et al.**, 1980; Barrett, 1972) for cathepsin B from different sources. pH stability experiments showed that the enzyme remains fully stable for at least 20 min upto pH 7.0 (Fig. 16). The enzyme was also found to have a maximum activity at physiological temperature and was fairly stable and retained most of its activity till the temperature was raised above 40°C (Fig. 17).

The activity of the enzyme seemed to be highly dependent on the ionic strength of the assay mixture (Fig.18). The ionic strength (0.022) at which maximal activity is shown by the enzyme happens to be the physiological ionic strength for many systems. It can thus be suggested that cathepsin B activity can persist for a limited period which could cause proteolysis even under physiological conditions before the enzyme is inactivated. The enzyme was found to be irreversibly inactivated at temperature and pH levels above physiological values. This may be due to the involvement of histidine imidazole group(s) in the catalytic process (Sumner **et al.**, 1993; Hasnain **et al.**, 1992). The irreversible inactivation of the enzyme at alkaline pH is probably because of deprotonation of active site His 199, which is brought about by OH^- ions, resulting in the breaking of the thiolate-imidazolium ion pair which further influences ionization and solvent exposure of some charged residues. This then critically destabilizes the enzyme and leads to unfolding of the enzyme (Turk **et al.**, 1994). The irreversible inactivation of the enzyme preparation at an alkaline pH also ruled out the possibility of cathepsin S contamination, since cathepsin S shows appreciable stability at alkaline pH (McDonald **et al.**, 1993; Kirschke **et al.**, 1989).

A characteristic feature of cathepsin B is its ability to inactivate aldolase, which also differentiated it from other cathepsins such as cathepsin H, L, S etc. However, only about 20% inhibition of aldolase activity was observed at aldolase/cathepsin B molar ratio of 50:1. 65% aldolase inactivation was reported when the molar ratio of aldolase to porcine liver cathepsin B was 100:1 (Takahashi **et al.**, 1986a). The results thus show that the goat enzyme is less effective towards aldolase inactivation than porcine cathepsin B and indicate a species difference in the choice of endogenous substrates.

Polyclonal antibodies raised against unresolved goat spleen cathepsin B cross-reacted distinctly with purified goat spleen enzyme preparations (i.e. unresolved goat spleen cathepsin B, GSCB-I & II) (Fig. 29 & 30). However, no precipitin line could be observed against buffalo kidney cathepsin B. Similarly when cathepsin H, a homologous enzyme of

lysosomal cysteine proteinases, either from buffalo kidney or from porcine lung was allowed to cross react with anti-goat spleen cathepsin B antibody, no precipitin line could be detected (Fig. 29). These observations pointed out that not only antigenic determinants are dissimilar between cathepsins B and H but also in cathepsin B from different species/tissues.

Cathepsin B from other sources have been shown to be capable of degrading soluble and insoluble collagen (Burleigh **et al.**, 1974), oxidized B-chain of insulin (Ansorge **et al.**, 1977), denatured hemoglobin (Agarwal & Khan, 1987b) and human fibrinogen (Guinec **et al.**, 1993) at acidic pHs. As evident from table IX, GSCB-I was found to be highly active against protein substrates tested. Out of BSA, hemoglobin and casein, hemoglobin (K_m = 1.46 μM) was found to be the most preferred substrate whereas BSA (K_m = 2.67 μM) was the least favoured one. The results thus suggest that the goat enzyme has a great potential to degrade proteins in vivo.

Among the N-blocked arginine derivatives, 2-napthylamide (BANA) and para-nitroanilide (BAPNA), the enzyme cleaved BAPNA most effectively. The K_m and V_{max}, obtained from double reciprocal plots were 0.07 mM and 0.03 units/mg protein for BAPNA and 2.64 mM and 0.51 units/mg protein for BANA, respectively. These values lie in the range of the values reported for the cathepsin B from other sources (Ahmad & Khan, 1990; Takahashi **et al.**, 1986; Fazili & Qasim, 1986; Barrett & Kirschke, 1981). A lower K_m for BAPNA compared to BANA may be explained possibly due to a better fit of nitroanilide of BAPNA in the S_1' pocket of catalytic site of the enzyme. Both the enzyme fractions (GSCB-I&II) showed markedly higher specificity towards fluorogenic substrates, Z-Arg-Arg-MCA and Z-Phe-Arg-MCA; the latter was found to be much more sensitive than the former (table XI). More sensitivity towards Z-Phe-Arg-MCA than Z-Arg-Arg-MCA is explained by the fact that specificity of papain like proteinases, in which cathepsin B falls, is largely determined by S_2-P_2 site interaction and such differences in the kinetic parameters suggest a probable difference in the topography corresponding to the positions S_1 and S_2 of the enzyme active site (Hasnain **et al.**,

1992; Baudys **et al.**, 1991; Musil **et al.**, 1991; Takahashi **et al.**, 1986b). It has been assumed that Arg is one of the best P_1 residue for cathepsin B because of the efficient hydrolysis of BANA and other arginine peptide amides (Takahashi **et al.**, 1986b). The specificity of S_2 pocket seems to favour large hydrophobic side chains such as phenylalanine since Cbz-Phe-Ala-CNH_2 is a potent inhibitor of cathepsin B (Green & Shaw, 1981; Watanabe **et al.**, 1979). Peptide amides with Arg at P_2 however seems to be less favoured compared to Phe (Takahashi **et al.**, 1986b). A similar order of substrate specificity was reported for mammalian, avian and piscean cathepsin B (Yamashita & Konagaya, 1990; Wada & Tanabe, 1988). However, larger K_m values of avian and piscean enzymes for Z-Arg-Arg-MCA and Z-Phe-Arg-MCA hydrolysis emphasize that goat spleen cathepsin B is more efficient towards fluorogenic substrates compared to avian/piscean enzymes and also reflect species difference between avian/piscean and mammalian enzymes.

A comparison of the results summarized above with that of the literature available on cathepsin B from other sources show that GSCB-I is similar with respect to most of its physicochemical properties with cathepsin B from other mammalian tissues. However, significant differences were seen in the amino acid composition and in its catalytic efficiencies towards various natural proteins and synthetic substrates where the V_{max} values were found to be higher. The K_m values for the various substrates were however, found to be well within the range reported earlier. The goat spleen cathepsin B showed much lower affinity for muscle aldolase compared to cathepsin B from other sources. This is a striking feature of this enzyme as aldolase is considered to be a good substrate for cathepsin B in general.

The differences, particularly in catalytic efficiency and the possible existence of isozymes of goat spleen cathepsin B clearly reflect a species and/or tissue dependence of the enzyme.

REFERENCES

Abrahamson, M., Barrett, A.J., Salvesen, G. and Grubb, A. (1986) J.Biol.Chem. 261, 11282-11289.

Abrahamson, M., Mason, R.W., Hansson, H., Buttle, D. J., Grubb, A. and Ohlsson, K. (1991) Biochem. J. 273, 621-626.

Achkar, C., Gong, Q., Frankfater, A., Bajkowski, A.S. (1990) J. Biol. Chem. 265, 13650-13654.

Agarwal. S.K. and Khan. M.Y. (1987a) Med. Sci. Res. 15, 387-388.

Agarwal, S.K. and Khan, M.Y. (1987b) Biochem. Int. 15, 785-792.

Agarwal, S.K. and Khan, M.Y. (1988) Biochem. J. 256, 609-614.

Agarwal, S.K. and Khan, M.Y.(1990) Biochem. Int. 15, 785-792.

Ahmad, S., Agarwal, S.K. and Khan, M.Y. (1989) J.Biosci. 14, 261-268.

Ahmad, S. and Khan, M.Y.(1990) Biochem.Int 22, 951-958.

Anderson. R.E., Woodbury, D.M. and Jee, W.S.S. (1986) Calcif. Tissue Int. 39, 252-258.

Andrews, P. (1970) in Methods in Biochemical Analysis, (Glick, D. ed.) Vol. XVIII, pp. 1-53, John Wiley and Sons, New York.

Ansorge, S., Kirschke , H. and Freidrick , K. (1977) Acta Biol. Med. Ger. 36, 1723-1727.

Bajkowski, A.S and Frankfater, A. (1983) J. Biol. Chem. 10, 1645-1649.

Banay-Schwartz, M., DeGuzman, T., Kenessey, A., Palkovits, M. and Lajtha, A. (1992) J. Neurochem. 58, 2207-2211.

Bando, Y., Kominami, E. and Katunuma, N. (1986) J. Biochem. 100, 35-42.

Baranski, T.J., Faust, P.L. and Kornfeld, S. (1990) Cell 63, 281-291.

Baranski, T.J., Koelsch, G., Hartsuck, J.A. and Kornfeld, S. (1991) J.Biol. Chem. 266, 23365-23372.

Baricos, W.H, Zhou, Y., Mason, R.W. and Barrett, A.J. (1988) Biochem. J. 252, 301-304.

Baricos, W.H., Cortez, S.L., Le, Q.C., Zhou, Y., Dicarlo, R.M., O'Connor, S.E., and Shah, S.V. (1990) Kid. Int. 38, 395-401.

Barka, T., Noen, H.V. and Patil, S. (1992) Lab. Invest. 66, 691-700.

Barrett, A.J. (1972) Anal. Biochem. 47, 280-293.

Barrett, A.J., (1973) Biochem. J. 131, 809-822.

Barrett, A.J. (1977) in Proteinases in Mammalian Cells and Tissues (Barrett, A.J. ed.), pp. 181-208. Amsterdam, North Holland.

Barrett, A.J. and Kirschke, H. (1981) Methods Enzymol. 80, 535-561.

Barrett, A.J., Kembhawi, A.A., Brown, M.A., Kirschke, H., Knight, C.G., Tamai, M., Hanada, K (1982) Biochem. J. 201, 189-198.

Barrett, A. J., Rawlings, N.D., Davies, M.E., Machleidt, W., Salvensen, G. and Turk, V. (1986) in Proteinase Inhibitors (Barrett, A.J. and Salvensen, G. eds) pp. 516-569. Elsevier Amsterdam.

Baudys, M., Meloun, B., Gan-Erdene, T., Pohl, J. and Kostka, V.(1990) Biol. Chem. Hoppe-Seyler. 371, 485-491.

Baudys, M., Meloun, B., Garendene, T., Fusek, M., Mares, M., Kestka, V., Pohl, J. and Blake, C.C.F. (1991) Biomed. Biochim. Acta 50, 569-577.

Berger, A. and Schechter, I. (1970) Philos. Trans. R. Soc. London B257, 249-264.

Bergman, M., Zerver, L. and Fruton, J.S., (1935) J. Biol. Chem. 111, 225-244.

Bergman, M. and Fruton, J.S. (1937) J. Biol. Chem. 118, 405-415.

Bio-Rad Protein Assay, Bio-Rad Technical Bulletin 1069, Bio-Rad Laboratories,

Richmond, C.A.

Bolli, R., Cannon, R.O., Spier, E., Goldstein, R.E. and Epstein, S.E. 1983) J. Am. Coll. Cardiol. 2, 681-688.

Bohley, P. and Seglen, P.O. (1992) Experientia (Basel) 48, 151-157.

Bond , J.S., Barrett, A.J. (1979) Fed. Proc. 39, 946-949.

Bradford, M. (1976) Anal. Biochem. 72, 248-254.

Bromme, D. and Kirschke, H. (1993) FEBS Lett. 322, 211-214.

Bromme, D., Bonneau, P.R., Lachance, P., Weideranders, B., Kirschke, H., Peters, C., Thomas, D.Y., Storer, A.C. and Vernet, T. (1993) J.Biol. Chem. 268, 4832-4838.

Buck, M.R., Karustis, D.G., Day, N.A., Honn, K.V. and Sloane, B.F. (1992) Biochem. J. 282, 273-278.

Burleigh, M.C., Barrett, A.J and Lazarus, G.S.(1974) Biochem. J.137, 387-398.

Chan, S.J., Segundo, B.S., McCormick, M.B. and Steiner, D.F. (1986) Proc. Natl. Acad. Sci. USA 83 , 7721-7725.

Corticchiato, O., Kajot, J.-F, Abrahamson, M., Chan, S. J., Kepler, D. and Sordat, B. (1992) Int. J. Cancer 52, 645-652.

Davidson, E., and Poole, B., (1975) Biochim. Biophys. Acta 397, 437- 422.

Davis, B.J. (1964) Ann. N.Y. Acad. Sci. 121, 404-427.

Docherty, K. Caroll,R.J. and Steiner, D.F. (1982) Proc. Natl. Acad. Sci. USA. 79, 4613-4617.

Docherty, K. and Phillips, I.D. (1988) Biochim. Biophys. Acta 964, 168-174.

Dolenc, I., Popovic, T., Cotic, V. and Turk, V. (1992) Eur. J. Clin. Chem. Clin. Biochem. 30, 69-74.

DuBois, M., Gilles, K.A, Hamilton, J.K., Roberts, P.A. and Smith, F. (1956) Anal. Chem. 28, 350-356.

Ellman, G.L. (1959) Arch. Biochem. Biophys. 82, 70-77.

Erdel, M., Trefz, G., Spiess, E., Habermoas, S., Spring, H., Lah, T. and Ebert, W.(1990) J. Histochem. Cytochem .38, 1313-1321.

Evans, E. and Shaw, E. (1983) J. Biol. Chem. 258, 10227-10232.

Fazili, K.M. and Qasim, M.A. (1986) J. Biochem. (Tokyo) 100, 293-299.

Ferrara, M., Wojcic, F., Rhaissi, H., Mordier, S., Roux, M.P and Bechet, D (1990) FEBS Lett. 273, 195-199.

Fok, A.K. and Paeste, R.M. (1982) Exp. Cell. Res. 139, 159-169.

Fong, D. Calhoun, D.H., Hsieh, W.T., Lee, B.and Wells, R.D. (1986) Proc. Natl. Acad. Sci. USA. 83, 2909-2913.

Fox,T., Miguel, Ede., Mort, J.S. and Storer , A.C. (1992) Biochemistry 31, 12571-12576.

Fruton, J.S. and Bergman, M. (1939) J. Biol. Chem. 127, 627-641.

Giordano, C., Gallina, C., Consalvi, V. and Scandurra, R. (1990) Eur. J. Med. Chem. 25, 479-487.

Giordano, C., Calabretta, R., Gallina, C., Consalvi, V., Scandurra, R., Noya, F.C., Franchini, C. (1993) Eur. J. Med. Chem. 28, 917-926.

Gong, Q., Chan, S.J., Bajkowski, A.S., Steiner, D.F. and Frankfater, A. (1993) DNA Cell Biol. 12, 299-309.

Gour-Salin, B.J., Lachance, P., Plouffe, C., Storer, A.C. and Menard, R. (1993) J. Med. Chem. 36, 720-725.

Gray, W.R. (1967) Methods Enzymol. 11., 139-151.

Green, G.D.J. and Shaw, E. (1981) J. Biol. Chem 256, 1923-1928.

Greenbaum, L.M. and Sherman, R. (1962) J. Biol. Chem. 237, 1082-1085.

Guinec, N., Dalet-Fumeron, V. and Pagano, M.(1993) Biol. Chem. Hoppe-Seyler 347, 1135-1146.

Hara, K., Kominami, E. and Katunuma, N. (1988) FEBS Lett. 231, 229-231.

Hashida, S., Towatari, T., Kominami, E., Katunuma. N. (1980) J. Biochem. 88, 1805-1811.

Hasnain, S., Hirama, T., Tam, A. and Mort, J.S. (1992) J. Biol. Chem. 267, 4713-4721.

Hasnain, S., Huber, C.P., Muir, A., Rowan, A.D. and Mort, J.S. (1992) Biol. Chem. Hoppe Seyler 373, 413-418.

Hasnain, S., Hirama, T. and Huber, C.P. (1993) J. Biol. Chem. 268, 235-240.

Howei, A.J., Burnett , D., Crocker, J. (1985) J. Pathol. 145, 307-314.

Huisman, W., Lating, Dodema, H.G., Bouma, J.M.W. and Grubes, M. (1974) Biochim. Biophys. Acta 370, 297-307.

Imperiali, B. and Abeles, R.H.(1986) Biochemistry 25, 3760-3767.

Jiang, S.T., Lee, J.J. and Chen, H.C. (1994) J. Agric. Food Chem. 42, 1073-1079.

Katunuma, N., Towatari, T., Taimai, M. and Handa, K., (1983) J.Biochem. (Tokyo) 93, 1129-1135.

Kenessey, A., Banay-Schwartz, M., DeGuzman, T., and Lajtha, A. (1989) J. Neurosci. Res 23, 454-456.

Keppler, D., Fondaneche, M.C., Dalet-Fumeron, V.,Pagano, M. and Burtin, P. (1988) Cancer Res. 48, 6855-6862.

Khan, M.Y., Ahmad, S. and Agarwal, S.K. (1986) IRCS Med. Sci. 14, 1141-1142.

Khan, M.Y., Agarwal, S.K. and Ahmad, S. (1992) J. Biochem. 111, 732-735.

Kirschke, H., Langer, J., Riemann, S., Wiederanders, B., Ansorge, S. and Bohley, P. (1980) in Protein Degradation in Health and Disease (D.Evered and J. Whelan, eds) pp. 15-35, Elsevier, Amsterdam.

Kirschke, H., Wood, L., Roisen, F.J. and Bird, J.W.C. (1983) Biochem. J. 214, 871- 877.

Kirschke, H., Weideranders, B., Bromme, D. and Rinne, A. (1989) Biochem. J. 264, 467-473.

Knight, C.G., (1980) Biochem. J. 189 447-453.

Kominami, E., Tsukahara, T., Bando, Y. and Katunuma , N. (1985) J. Biochem. 98, 87-93.

Kominami, E., Tsukuhara, T., Hara, K. and Katunuma, N. (1988) FEBS Lett. 231, 225-228.

Kornfeld, S. (1992) Annu. Rev. Biochem. 61, 307-330.

Laemmli, U.K. (1970) Nature (London) 227, 680-685.

Lah, T.T, Buck, M.R., Honn, V.K., Crissman, J.D., Rao, N.C., Loitta, L.A. and Sloane, B.F. (1989) Clin. exp. Metastasis 7, 461-468.

Laszlo, A., Sohar, I., Sagi, I., Kovacs, J. and Kovacs, A. (1993) Clin. Chim. Acta 210, 233-235.

Laurent, T.C. and Killander, J. (1964) J. Chromatogr. 14, 317-330.

Lineweaver, H. and Burk, O. (1934) J. Am. Chem. Soc. 56, 658-666.

Mach, L., Mort, J.S. and Glossl, J. (1994) J. Biol. Chem. 269, 13030-13035.

Maciewicz, R.A. and Etherington, D.J. (1988) Biochem. J., 256, 433-440.

Maciewicz, R.A., Wardale, R.J., Etherington, D.J., and Paraskeva, C. (1989) Int., J. Cancer 43, 478-486.

Maciewicz, R.A., Wotton, S.F., Etherington, D.J., Duance, V.C.(1990) FEBS Lett. 269, 189-193.

MacGregor, R.R., Hamilton, J.W., Shofstall, R.E. and Cohn, D.V. (1979) J. Biol. Chem. 254, 4423-4427.

MacGregor, R.R., Jilka, R.L. and Hamilton, J.W. (1986) J. Biol. Chem. 261, 1929-1934.

Mc Donald, J.K. and Ellis , S. (1975) Life Sci. 17, 1269-1276.

McDonald, J. K., Culbertson, J.T. and Owers, N.O. (1993) Arch. Biochem. Biophys. 305, 1-8.

McKay, M.J., Offermann, M.K., Barrett, A.J. and Bond, J.S. (1983) Biochem. J. 213, 467-471.

Martinek, R.G., Berger, L. and Broida, D. (1964) Clin. Chem. 10, 1087-1091.

Medhi, S., Angelastro, M.R., Burkhart, J.P., Koehl, J.R., Peet, N.P. and Bey, P. (1990) Biochem. Biophys. Res. Commun. 166, 595-600.

Medhi, S. (1991) Trend Biochem. Stud. 16, 150-153.

Meloun, B., Baudys, M., Pohl, J., Pavlik, M. and Kostka, V., (1988) J. Biol. Chem. 263, 9089-9093.

Mizuno, K., Miyata, A., Kongawand, K. and Matsuo, H. (1982) Biochem. Biophys. Res. Commun. 108, 1235-1242.

Montenez, J.P., Delaisse, J.M., Tulkens, P.M. and Kishore, B.K. (1994) Life Sci. 55, 1199-1208.

Moore, S. and Stein, W.H. (1954) J. Biol. Chem. 211, 907-913.

Mordier, S., Bechet, D., Roux, M., Obled, A. and Ferrara, M. (1993) Biochim. Biophys. Acta 1174, 305-311.

Mort, J.S., Recklies, A.D., and Poole, A.R. (1980) Biochim. Biophys. Acta 614, 134-142.

Mort, J.S., Leduc, M.S., and Recklies, A.D., (1983) Biochim. Biophys. Acta 755, 369-375.

Mort, J.S., Recklies, A.D., Poole, A.R. (1984) Arthritis Rheum. 27, 509-515.

Muno, D., Sutoh, N., Watanabe, T., Uchiyama, Y. and Kominami, E. (1990) Eur. J. Biochem. 191, 91-98.

Musil, D., Zucic, D., Turk, D., Engh, R.A., Mayr, I., Huber, R., Popovic, T., Turk, V., Towatari, T., Katunuma, N. and Bode, W. (1991) EMBO. J. 10, 2321-2330.

Nakai, N., Wada, K., Kobashi, K. and Hase. J. (1978) Biochem. Biophys. Res. Commun. 83, 881-885.

Narita, K. (1970) in Protein Sequence Determination, (Needleman, S.B. ed.), pp. 25-90, Chapman and Hall Ltd. London.

Natowicz, M., Baenziger, J.U. and Sly, W.S. (1982) J. Biol. Chem. 257, 4412-4420.

Neurath, H. (1955) Methods Enzymol. 2, 77-83.

Nishimura, Y. and Kato, K. (1987) Biochem. Biophys. Res. Commun. 148, 254-259.

Nishimura, Y., Amano, J., Sato, H., Tsuji, H., and Kato, K.(1988) Arch. Biochem. Biophys. 262, 159-270.

Nishimura, Y., Kawabata, T., Yano, S. and Kato, K. (1990) Acta Histochem. Cytochem. 23, 53-64.

Otto, K. (1971) in Tissue Proteinases (Barrett, A.J. and Dingle, J.T. eds.) pp. 1-28 North-Holland, Amsterdam.

Ouchterlony, O. (1949) Acta Pathol. Microbiol. Scand. 26, 507-515.

Peet, N.P., Burkhart, J.P., Angelastro, M.R., Giroux, E.L., Medhi, S., Bey, P., Kolb, M., Neises, B. and Schirlin, D. (1990) J. Med. Chem. 33, 394-407.

Pietras, R.J. and Roberts, J.A. (1981) J.Biol. Chem. 256, 8536-8544.

Porath, J. (1963) Pure Appl. Chem. 6, 233-244.

Qian, F., Frankfater, A., Miller, R., Chan, S.J. and Steiner, D.F. (1990) Int. J. Biochem. 22, 1457-1464.

Quinn, P.S. and Judah, J.D. (1978) Biochem. J. 172, 301-309.

Recklies, A.D., Poole, A.R. and Mort, J.S. (1982) Biochem. J. 207, 633-636.

Rhaissi, H., Bechet, D. and Ferrara, M. (1993) Biochimie 75, 899-904.

Ritonja, A., Popovic, T., Turk, V., Widenmann, K. and Machleidt, W. (1985) FEBS Lett. 181, 169-172.

Rozhin, J., Wade, R.L., Honn, K.V., Sloane, B.F. (1989) Biochem. Biophys. Res. Commun. 164, 556.

Sano, K., Waguri, S., Sato, N., Kominami, E. and Uchiyama, Y., (1993) J. Histochem Cytochem. 41, 433-438.

Schecter, I. and Berger, A. (1967) Biochem. Biophys. Res. Commun. 27, 157-162.

Schecter, I. and Berger, A. (1968) Biochem. Biophys. Res. Commun. 32, 898-902.

Shaw, E. and Dean, R.T. (1980) Biochem. J. 186, 385-390.

Sheahan, K., Shuja, S. and Murnane, M. J.(1989) Cancer Res. 49, 3809-3814.

Sher, J.H., Stracher, A., Shafiq, S.A. and Hardy-Stashin, A. (1981) Proc. Natl. Acad. Sci. USA 79, 7742-7744.

Singh, H. and Kalnitzky, G. (1978) J.Biol.Chem. 253, 4319-4326.

Sloane, B.F. (1990) Semin. Cancer Biol. 1,137-152.

Sloane, B.F., Buck, M.R., Karustis, D.G., Day, N.A. and Honn, K.V. (1992) Biochem. J. 282, 273-278.

Sohar, I. and Gyorgy, K. (1992) Biol. Chem. Hoppe-Seyler 373, 567-572.

Spiess, E., Erdel, M., Trefz, G., Habermass , S., Spring ,H., Lah, T., Ebert, W. (1990) J. Histochem. Cytochem. 38 ,1313-1321.

Spiess, E., Bruning, A., Gack, S., Ulbricht, B., Spring,H., Trefz, G. and Ebert, W. (1994) J. Histochem. Cytochem. 42, 917-929.

Shaw, E. and Dean, R.T., (1980) Biochem. J. 186, 385-390.

Stein, R.L., Strimpler, A.M., Edwards, P.D., Lewis, J.J., Mauger, R.C., Schwartz, J.A., Stein, M.M., Trainor, D.A., Wildonger, R.A. and Zottola, M.A., (1987) Biochemistry 26, 2682-2689.

Steiner, D.F., Docherty, K., Chan, S.J., Segundo, S.B. and Carroll, B. (1983) in Biochemical and Clinical Aspects of Neuropeptides: Synthesis, Processing, and Gene Structures, (G. Koch and D. Richter., eds) 3-13 Acamedic Press, New York.

Stracher, A., McGowan, E.B. and Shafiq, S.A. (1978) Science 200, 50-51.

Sumiya, S., Yoneda,T., Kitamura, K., Murata, M., Yokoo, C., Tamai, M., Yamamoto, A., Inoue, M., Ishida, T.(1992) Chem. Pharma. Bull. 40, 299-303.

Sumner, I.G., Vaughan, A., Eisenthal, R., Pickergill, R.W., Owen, A.J. and Goodenough, P.W. (1993) Biochim. Biophys. Acta 1164, 243-251.

Takahashi, T., Schmidt, P.G. and Tang, J. (1983) J. Biol. Chem. 258, 2819-2830.

Takahashi, T., Dehdarani, A.H., Schmidt, P.G. and Tang, J. (1984a) J. Biol. Chem. 259, 9874-9882.

Takahashi, T., Schmidt, P.G. and Tang, J. (1984b) J. Biol.Chem. 259, 6059-6062.

Takahashi, T., Yonezawa, S., Dehdarani, A.H. and Tang, J. (1986a) J. Biol.Chem. 261, 9368-9374.

Takahashi, T., Dehdarani, A.H., Yonezawa, S and Tang, J (1986b) J. Biol. Chem. 261, 9375-9381.

Takio, K., Towatari, T., Katunuma, N., Teller, D.C. and Titani, K. (1983) Proc. Natl. Acad. Sci. U.S.A. 80, 3666-3670.

Tallen, H.H., Jones, M.E., and Fruton, J.S. (1952) J. Biol. Chem. 114, 793-805.

Tamai, M., Hanada, K., Adachi, T., Oguma, K., Kashiwagi, K., Omura. S., Ohzeki, M. (1981) J. Biochem. 90, 255-257.

Tanaka, K., Ikegaki, N. and Ichihara, A.(1984) J. Biol. Chem. 259, 5937-5944.

Tanford, C. (1955) J. Phys. Chem, 59, 789-799.

Tanford, C. (1961) in Physical Chemistry of Macromolecules, John Wiley and Sons, New York.

Tanford, C. (1968) Adv. Protein Chem. 23, 121-282.

Taugner, R., Buhrle, C.P., Nobiling, R. and Kirschke, H.(1985) Histochemistry 83, 103- 108.

Teale, F.W.J. (1960) Biochem. J. 67, 381-388.

Thompson, R.C. (1973) Biochemistry 12, 47-51.

Tomomasa, H., Waguri, S., Umeda, T., Koiso, K., Kominami, E. and Uchiyama, Y. (1994) J. Histochem. Cytochem. 42, 417-425.

Trabandt, A., Gay, R.E., Fassbender, H.G. and Gay, S. (1991) Arthritis Rheum. 34, 1444-1451.

Turk, B., Dolenc, I., Zerovnik, E., Gubensek, F. and Turk, V. (1994) Biochemistry 33, 14800-14806.

Uchiyama, Y., Watanabe, T., Watanabe, M., Ishii, Y., Matsuba, H., Waguri, S., and Kominami, E. (1989) J. Histochem. Cytochem. 37, 691-696.

Uchiyama, Y., Nakajima, M., Muno, D., Watanabe, T., Ishii, Y., Waguri, W., Sato, N. and Kominami, E. (1990) J. Histochem. Cytochem. 39, 633-639.

Uchiyama, Y., Nakajima, M., Watanabe, T., Waguri, S., Yamamoto, M., Hashizume, Y. and Komonami, E. (1991) J.Histochem. Cytochem. 39. 1199-1205.

Uchiyama, Y., Waguri, S., Sato, N., Watanabe, T., Ishido, K. and Kominami, E. (1994) Acta. Histochem. Cytochem. 27, 287-308.

Van Noorden, C.J.F., Vogels, I.M.C. and Smith, R.E. (1989) J. Histochem. Cytochem. 37, 617-624.

Von Figura, K. and Hasilik, A. (1986) Annu. Rev. Biochem. 55, 167-193.

Wada, K. and Tanabe, T. (1988) J. Biochem. 104, 472-476.

Walker, B., McCarthy, N., Healy, A., Ye, T. and McKervey, M.A. (1993) Biochem. J. 293, 321-323.

Watanabe, H., Green, G.D.J. and Shaw, E. (1979) Biochem. Biophys. Res. Commun. 89, 1354-1360.

Watanabe, T., Waguri, S., Watanabe, M., Ishii, Y., Kominami, E. and Uchiyama, Y. (1989) J. Histochem . Cytochem. 37 , 1899-1911.

Weinhouse, S., Shatton, J.B. and Morris, H.P. (1976) in Cancer Enzymology (Schultz, J. and Ahmad, F., eds) 41-61, Academic Press, New York.

Westerik, J.O. and Wolfenden , R. (1972) J. Biol. Chem. 247, 8195-8197.

Willenbrock, F. and Brockleharst, K.(1985) Biochem.J. 227, 521-528.

Willstatter, R. and Bamann, E. (1929) Z. Physiol. Chem. 180, 127-143.

Yamamoto, K., Kamata, O. and Kato, Y. (1984) Japan. J. Pharmacol. 35, 253-258.

Yamamoto, Y. and Takahashi, S.Y. (1993) Comp. Biochem. Physiol. 106, 35-45.

Yamashita, M. and Konagaya, S.(1990) Comp. Biochem. Physiol. 96B, 733-737.

Young, P.R. and Spevacek, S.M. (1993) Biochim. Biophys. Acta 1182, 69-74.

Zvonar-Popovic, T., Lah, T., Kregar, I. and Turk, V. (1980) Croat. Chem Acta. 53, 509-517.