STUDIES ON THE PHYSICO - CHEMICAL AND BIOLOGICAL PROPERTIES OF BUFFALO *(Bubalus bubalis)* KIDNEY CATHEPSIN B.

By

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THESIS

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CERTIFICATE

Certified that the thesis entitled "STUDIES ON THE PHYSICO-CHEMICAL AND BIOLOGICAL PROPERTIES OF BUFFALO *(Bubalus bubalis)* KIDNEY CATHEPSIN B" submitted by Mr. **Madhab Lamsal** for the award of the degree of DOCTOR OF PHILOSOPHY in Biochemistry of the North-Eastern Hill University Shillong, embodies the record of original investigations carried out by him under my 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 Univesity.

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ABSTRACT

Owing to their ubiquitous distribution in biological tissues and fluids and probable participation in different physiological and pathological processes, proteolytic enzymes have been the subject of critical investigation for the past few decades. It is now well established that all the lysosomal proteinases characterized till date belong to one or other of the four classes: cysteine, aspartic, serine or metalloproteinases. Cysteine proteinases, characterized by the presence of an essential cysteine residue at the active site, are involved in lysosomal as well as extra-lysosomal protein degradation and turnover. A number of proteinases with acidic pH optima, known as "cathepsins" are found in the lysosomal system. Of these, cathepsins B,H,L,S and M of the cysteine group, cathepsin D of aspartyl group and cathepsin G of the serine group constitute the chief proteinases which have been studied in details.

Cathepsin B (EC 3.4.22.1) is the most well characterized lysosomal cysteine proteinase belonging to the papain superfamily. Under normal conditions, this enzyme participates in vital physiological processes that involve cellular protein turnover. However, in cases where the enzyme becomes deregulated, cathepsin B has been shown to be associated with a number of diseased states.

In the present study, a simple procedure for simultaneous purification of cathepsins B and H from buffalo kidney has been developed. Purified buffalo kidney cathepsin B was found to be homogeneous on the basis of charge as well as size.

Molecular weight of the purified enzyme both from gel filtration and SDS-PAGE studies was found to be very close (26 kDa) to what has been reported for this enzyme from other sources. The stokes radius was calculated to be 2.32. However, electrophoretic studies both in absence and presence of SDS resulted into single prominent protein band suggesting that the enzyme lacked subunit structures and/or isozymes.

Buffalo kidney cathepsin B had an isoionic pH of 5.1, which was well within the range (4.8-5.3) reported for this enzyme from other sources.

One of the striking differences between buffalo kidney cathepsin B and those isolated from other sources was the presence of alanine as the NH_2- terminal amino acid residue in the former as against leucine in others. However, the COOH-terminal amino acid residue was found to be threonine which was the same as reported for cathepsin B from other tissues/sources. This may either be attributed to simple species dependence or more significantly to the possible differential post translational processing of the enzyme in the kidney tissues.

The total free sulfhydryl groups contents were found to be

0.6 and 1.6 mol/ mol of proteins under native and denaturing conditions respectively, suggesting their partial burial under the native conditions.

The glycoprotein nature of the enzyme was confirmed with the total content of about 3.6 (glucose equivalent). This value was significantly lower than what is found in cathepsin B from buffalo spleen or porcine spleen and could possibly be responsible for the altered hydration and catalytic and immunological characteristics of the buffalo kidney enzyme.

Amino acid composition of buffalo kidney cathepsin B showed close similarities with its counterparts from other sources like bovine, porcine, human and rat tissues with an exception to serine. The extinction coefficient ($E^{1\%}_{1cm}$) for this enzyme was found to be 16.78, which was comparable to the values determined for the enzyme from other sources.

Like its counterparts from other sources/tissues, buffalo kidney cathepsin B was found to be very sensitive to parameters like temperature, pH, ionic strength and radiation. The enzyme showed maximum activity near the physiological levels of pH (6.8) and temperature (40°C). One of the striking characteristics of the kidney enzyme was that it underwent reversible inactivation at higher salt concentration or buffer strength. Hence for most enzymatic assays, the buffer strength was restricted to 20 mM, while excess salt was used for the long term storage of the

enzyme. Low doses of gamma-irradiation (2-20 Gy) had a stimulatory effect on the enzyme, whereas, higher doses (>30 Gy) led to significant loss in its activity.

Like any other cysteine proteinases, the buffalo kidney enzyme required presence of thiol reducing agents to express its optimal catalytic activity. Among the various thiol reducing agents tested, DTT and cysteamine-HCl were found to be the most and the least effective respectively. The thiol blocking compounds such as $HgCl_2$ and $ZnSO_4$, alkalyting agents like iodoacetic acid and iodoacetamide, and peptidyl inhibitors like leupeptin, antipain, chymostatin and E-64 acted as potent inhibitors of the enzyme. Pepstatin, a well known inhibitor of aspartyl proteinases, including cathepsin D, on the other hand was found to be ineffective towards the buffalo enzyme.

Denaturants such as urea and Gdn-HCl had profound effect on the activity of the kidney enzyme. More than 50% of the enzyme activity was lost at the urea concentrations >1.0 M and virtually no activity was found above 3.0 M. Reactivation studies showed that the enzyme activity could be regained only partially. This in consistence to what has been reported for its counterparts from liver and spleen. In addition, it also rules out the possibility of contamination of our cathepsin B preparation with cathepsin L.

Gdn-HCl was highly effective against the kidney cathepsin B

and it suppressed more than 50% of the enzymatic activity at a concentration as low as 0.01 M. The inactivation of the enzyme was found to be reversible for Gdn-HCl exposure up to 0.1 M. Urea and Gdn-HCl induced inactivation studies of buffalo kidney cathepsin B taken together, therefore suggest that although the concentrations of those denaturants required to fully inactivate the enzyme were too low to cause any major change in the gross structure of the enzyme, yet, the possibility of some minor "structural perturbations" around the enzyme active site effecting its activity is not ruled out.

The results on the kinetic studies of cathepsin B from buffalo kidney indicated that the enzyme has great catalytic potential against various synthetic as well as protein substrates at acidic/ or near neutral pHs. Among the four synthetic substrates tested, Z-Phe-Arg-MCA with K_m of 0.0909 mM was found to be the most preferred followed by Z-Arg-Arg-MCA (0.166 mM), BAPNA (0.1818 mM) and BANA (3.33 mM). No activity could be observed against Arg-NA, Leu-NA or Arg-MCA. These K_m were well within the range available for cathepsin B from other sources. However, the corresponding V_{max} values differed drastically from what is reported for cathepsin B from other sources.

Goat and buffalo haemoglobins with the corresponding K_m values of 1.428 μM and 2.173 μM respectively, were found to be the most preferred protein substrates for buffalo kidney cathepsin B.

Immunological studies showed that antibodies could be raised in rabbits against purified and mature buffalo kidney cathepsin B that recognizes both native as well as pH denatured cathepsin B from the buffalo kidney source. Thus, the anti-buffalo kidney cathepsin B antibody could be used as an effective tool to distinguish the enzyme from other related proteinases like cathepsins H and L from same or different sources.

A comparison of the above data on buffalo kidney cathepsin B with published results on cathepsin B from other sources thus reveals that buffalo kidney enzyme is similar to its counterparts from other sources with respect to (i) Molecular weight, (ii) hydrodynamic properties, (iii) catalytic nature, (iv) response to inhibitors and (v) optical properties, but, differ significantly with regards to its (1) NH_2- terminal amino acid residue, (2) carbohydrate contents, (3) serine contents, (4) catalytic efficiencies against various synthetic peptide and natural protein substrates including muscle aldolase, (5) immunological properties and lack of multiple chain form and/or isozyme. All these findings taken together therefore, suggests a strong species and/or tissue dependence of cathepsin B in mammalian sources.

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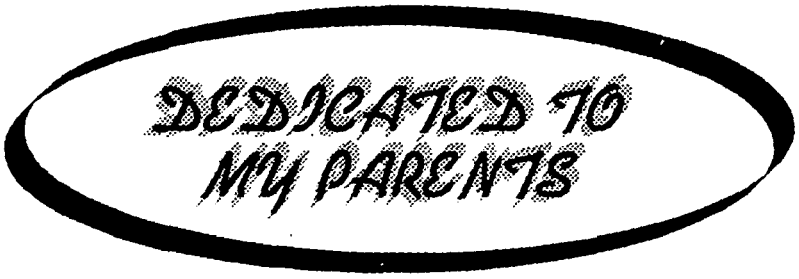
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LIST OF ABBREVIATIONS

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
BZ-Arg-2-Nap	: Benzoyl-arginine-2-napthylamide
CM	: Carboxymethyl
CPI	: Cysteine proteinase inhibitor
DEAE	: Diethylaminoethyl
DFP	: Diisopropyl phosphofluoridate
DMSO	: Dimethylsulfoxide
DTNB	: 5,5'-dithio-bis-(2-nitrobenzoic acid)
DTT	: Dithiothreitol
EDTA	: Ethylene diamine tetra acetic acid
E-64	: trans-epoxy-succinyl-L-leucylamino(4-guanidino)-butane
EP-475	: trans-epoxy-succinyl-leucylamido(3-methyl)-butane
FCA	: Freund's complete adjuvent
Gdn-HCl	: Guanidine hydrochloride
HPLC	: High Performance Liquid Chromatography
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 electrophoresis
PB-74	: Poly buffer 7-4
PBE-94	: poly buffer exchanger 9-4
PBS	: Phosphate buffer saline
pCMB	: p-Chloromercuribenzoate
pHMB	: p-Hydroxy-chloromercuribenzoate
R_f	: Relative front
R_m	: Relative mobility
SDS	: Sodium dodecyl sulphate
TCA	: Trichloro acetic acid
TEMED	: N,N,N',N',-tetramethylethylenediamine
TLC	: Thin layer chromatography
TPI	: Thiol proteinase inhibitor
Tris	: Tris-(hydroxymethyl)-aminomethane
Z	: Benzyloxycarbonyl
Z-Arg-Arg-MCA	: N-α-benzyloxycabonyl-L-arginyl-L-arginine-7-amido-4-methylcoumarin
Z-Phe-Arg-MCA	: N-α-benzyloxycarbonyl-phenylalanyl-L-arginine-7-amido-4-methylcoumarin
Z-Phe-Ala-CHN_2	: Benzyloxycarbonyl-(phenylalanyl-alanine)-diazomethane
Z-Phe-Phe-CHN_2	: Benzyloxycarbonyl-(phenylalanyl-phenylalanine)-diazomethane

STUDIES ON THE PHYSICO-CHEMICAL AND BIOLOGICAL PROPERTIES OF BUFFALO (*Bubalus bubalis*) KIDNEY CATHEPSIN B

INTRODUCTION

The importance of mammalian proteinases was first recognized by Salkowski in 1890 and since then, studies on tissue proteinases have gained considerable significance (Barrett, 1981; Scott, 1992). Owing to their wide range participation in vital physiological processes including peptide biosynthesis and protein degradation substantial progress has been made in the field of isolation and characterisation of proteolytic enzymes during the past few decades (Barrett **et al.**, 1981; Kirschke **et al.**, 1983; Tanaka **et al.**1984; Taugner **et al.**, 1985; Scott 1992;Sohar **et al.**, 1992; Yamamoto and Takahashi,1993).

Lysosomes, which were once termed as "suicidal sacs" are looked upon at present as the store house of various clinically important enzymes with immense catalytic potential (DeDuve, 1983; Katunuma, 1973). Intralysosomal protein degradation is a composite process that is largely controlled by a battery of proteinases (Galjart **et al.**, 1991; Sohar **et al.**, 1992). Cathepsins constitute the major group of proteinases in the lysosomes of most mammalian cells. The term Cathepsin (Latin "KATHEPSIN") meaning " to digest" was first coined by Willstatter and Bamman (1929) for the aqueous extract of a variety of mammalian tissues exhibiting proteinase activity in the acidic range.

Fruton and Bergmann (1939) identified three types of cathepsins depending on their specificity against low molecular weight peptide substrates, viz., Z-Glu-Tyr, BZ-Arg-NH_2 and BZ-leu-NH_2 (where Z and BZ are carbonyl and benzoyl groups re-

spectively), and designated them as cathepsins I, II and III. Reclassification of these three cathepsins was done by Tallen **et al.,** (1952) as cathepsin A, B and Leucine aminopeptidase respectively. Another enzyme active against Z-Glu-Phe was also identified by Tallen **et al.,** (1952), which turned out to be a thiol dependent caboxypeptidase and was designated as cathepsin IV or cathepsin C. Besides these, two other peptide depending enzymes were identified as Cathepsins D and E (Greenbaum **et al.,** 1962).

With the availability of a board spectrum of substrates and inhibitors of great specificity, more than a dozen cathepsins and related proteinases have been identified and characterised till date. However, due to their overlapping specificities, mode of action and homologous active site, the exact roles of these enzymes in pathophysiological processes still remains speculative.

It is now established that all major proteinases of lysosomal origin belong to one or other of the four families: Cysteine, Aspartic, Serine or Metalloproteinases. The classification is based on the functional criteria, namely, the nature of the most prominent functional group in the active site (Yamamoto **et al.,** 1993).

Cysteine proteinases characterised by the presence of an essential cysteine residue at their active site form the chief constituent of lysosomal cathepsins (Barrett **et al.,** 1973). The most notable of these proteinases include cathepsin B (EC 3.4.22.1), cathepsin L (EC 3.4.22.15), cathepsin H (EC 3.4.22.16)

and cathepsin S (EC 3.4.22.27). These enzymes were identified by their ability to degrade small peptides (Tab. I) and their broad tissue distribution. This is in consistence with their role as general protein degrading enzymes (Dellaisse **et al.,** 1991; Yamamoto and Takahashi, 1993; McDonald **et al.,** 1993; Guinec **et al.,** 1993). Studies of the primary structures of these enzymes show substantial sequence homology with papain (a well characterised plant cysteine proteinase), especially around the active site triad and hence these are grouped under "Papain superfamily" (Kirschke **et al.,** 1983; Tanaka **et al.,** 1983; Takio **et al.,** 1983; Takahashi **et al.,** 1984a, 1986; Baudys **et al.,** 1991; Dolenc **et al.,** 1992; Mordier **et al.,** 1993). Besides these well characterised cathepsins, several other thiol proteinases like cathepsins I, J, K, M, N, P and T have also been isolated from various sources. However, due to their somewhat overlapping substrate specificity and very low content, the characterisation of these enzymes is still in their infancy (Pontremoli **et al.,** 1982; Maciewiez **et al.,** 1988; Krischke **et al.,** 1989).

Cathepsin B is the most thoroughly studied proteinases among the thiol cathepsins. The enzyme originally identified for its ability to hydrolyse BZ-Arg-NH_2 in presence of cysteine, has now been isolated and characterised from numerous sources showing its broad tissue distribution. The enzyme shows both exopeptidase and endopeptidase activities with trypsin like substrate specificity. The enzyme also shows a dipeptidyl carboxypeptidase activity, which is unique to cathepsin B among all the cysteine

TABLE I : Cysteine Proteinase Substrates

Assay substrates	Lysosomal cysteine proteinase		
	Cathepsin B	Cathepsin H	Cathepsin L
BZ-Arg-NA	+	+	-
Z-Arg-Arg-NA	+	-	-
Arg-NA	-	+	-
Azocasein	+	+	+
Z-Arg-Arg-MCA	+	-	-
Arg-MCA	-	+	-
Z-Phe-Arg-MCA	+	-	+
Z-Phe-Arg-MCA + Z-Phe-Phe-CHN_2	+	-	-

Adopted from Barrett, A.J. (1977).

proteinases (Takio **et al.,** 1983; Bajkowski and Frankfater,1983; Evans and Shaw 1983; Baudys **et al.,** 1991). The requirement of thiol activators, low pH optima and the inhibition of the activity in presence of thiol group blocking compounds supports its classification among the cysteine proteinases (Shaw **et al.,** 1983; Takio **et al.,** 1983; Kamphuis **et al.,** 1985; Dufour 1988; Hempel **et al.,** 1991; Baudys **et al.,** 1991).

LOCALIZATION :

Sensitive histochemical and immunohistochemical studies have confirmed the localization and expression of lysosomal cathepsin B in a large varieties of tissues including kidney, spleen, liver, heart, bone, thyroid, brain, duodenum, renal tubules, bladder, breast, cervix, vagina, placenta, lungs, prostate etc., and also from a number of benign and metastatic carcinomas (Yokota **et al.,** 1986; Sinha **et al.,** 1989; Cornelis **et al.,** 1989; Bernstein **et al.,** 1989; Uchiyama **et al.,** 1989; Krepela **et al.,** 1989; Erdel **et al.,** 1990; Barka **et al.,** 1992).

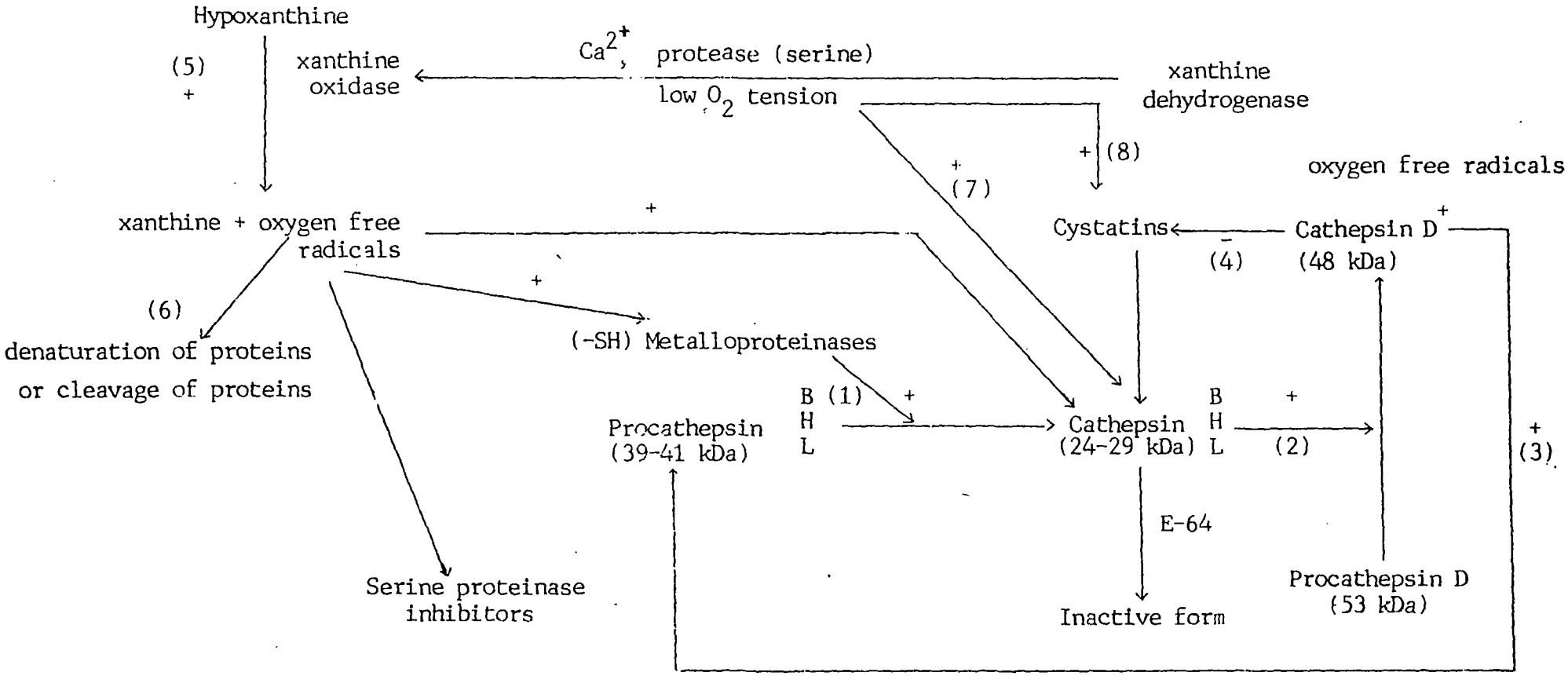
BIOSYNTHESIS AND PROTEOLYTIC PROCESSING :

Cathepsin B is synthesized on the lumen of rough endoplasmic reticulum. Like many other lysosomal cathepsins, it is synthesized as a larger molecular mass precursor, which is latent. The latent pre-procathepsin is subsequently transported to the smooth endoplasmic reticulum and golgi apparatus before it is targeted to lysosomes or secreted in the extra cellular fluids (Barrett,1977). Proteolytic maturation of the latent pro-enzyme

to the active enzyme occurs either in an acidic prelysosomal compartment (Gisselman **et al.,** 1983; Grinde **et al.,** 1985;Fox **et al.,** 1992; Mach **et al.,** 1993), or as an early event in prelysosomes (Nishimura **et al.,** 1987) and possibly regulated by a series of complex reactions (Fig. 1).

Existence of lysosomal cathepsins into their proenzyme or zymozen forms have been well documented. (Segundo **et al.,** 1985; Qian **et al.,** 1991; Page **et al.,** 1992). Characterization of nucleotide sequence of cDNA encoding human, mouse, rat and bovine cathepsin B revealed that this enzyme is synthesized as a preproenzyme (Segundo **et al.,** 1985; Chan **et al.,** 1986; Fong **et al.,** 1986; Ferrara **et al.,** 1990; Qian **et al.,** 1991; Mordier **et al.,** 1993). Cathepsin B from human , rat and mouse is synthesized as a single chain of 339 amino acid residues as against 335 in case of bovine. These are processed to the mature single chain of 254 and 253 residues by cleaving off 79 residues propeptides and 6 and 3 residues as C- terminal processing in the former and the latter respectively. Further processing of single chain mature cathepsin B into the two chain form occurs by endoproteolytic cleavage of the residues 48-49, giving rise to 47 (or 49) residues light chain and 205 (or 204) residues heavy chain. The two chains are linked to each other by a single covalent disulfide bridge (Musil **et al.,** 1991; Mordier **et al.,** 1993). Inhibitor studies showed that the conversion of single chain form to two chain form could be inhibited **in vivo** by leupeptin, a potent reversible cysteine proteinase inhibitor. (Mach **et al.,** 1992).

Fig. 1. Regulation of proteinase activation in mammalian tissues (Sohar **et al.**, 1992).



(1) K. Hara et al. 1988 - cultured rat peritoneal macrophages
(2) A.M. Samarel et al. 1986 - rabbit cardiac muscle
(3) Y. Nishimura et al. 1988 - primary cultures rat hepatocytes
(4) B. Lenarnic et al. 1988 - in vitro
(5) D.A. Parks et al. 1983 - digestive tract
(6) R. Dean 1987 - in vitro
(7) T. Kooistra et al. 1982 - in vitro
(8) T. Tsukahara et al. 1987 - marcophage cell culture

Cathepsin B is a glycoprotein and it has been found that variation in the mode of glycosylation has some bearing on the targeting of this enzyme to lysosomes through the mannose-6-phosphate dependent transport system (Dahms **et al.,** 1989). The carbohydrate moieties are mostly present in the heavy chain and are linked with Asn-111 (or Asn-113) (Takio **et al.,** 1983; Musil **et al.,** 1991).

Heterogeneity of glycoproteins obtained from different tissues has been attributed frequently to cell type specific events in glycosylation (Rademacher **et al.,** 1988; Meloun **et al.,** 1988; Mach **et al.,** 1992) and could be accounted for the possible Cathepsin B isozymes (Takahashi **et al.,** 1986). However, the exact functions of these differently glycosylated isozymes still remain obscure.

PHYSICAL CHARACTERISTICS :

(A) Molecular weight :

Cathepsin B is a globular glycoprotein. The enzyme isolated from different sources have been found to have wide variations in relation to its molecular weight. A molecular weight of 22 kDa to 32 kDa has been assigned to this enzyme from the normal cells and tissues. However, the enzyme from a number of malignant and tumour cells and tissues is confirmed to be present in multiple isoforms with molecular weight ranging from 20 kDa to 39-46 kDa. The higher molecular weight forms have been attributed to the presence of latent pre or pro enzyme forms. (Qian **et al.,** 1989;

Rozhin **et al.,** 1990, Delaisse **et al.,** 1991, Tanabe **et al.,** 1991; Matsuoka **et al.,** 1992; Page **et al.,** 1992, Mach **et al.,** 1993).

Cathepsin B from bovine, porcine, mouse and human tissue sources are found to be present in two isoforms: single chain form with a molecular weight of about 26 kDa to 29 kDa and two chains form with the heavy chain of 22-24 kDa and the light chain of about 4-5 kDa. The light chain is linked to the heavy chain by a single disulfide bridge (Musil **et al,** 1991, Baudys **et al,**1991).

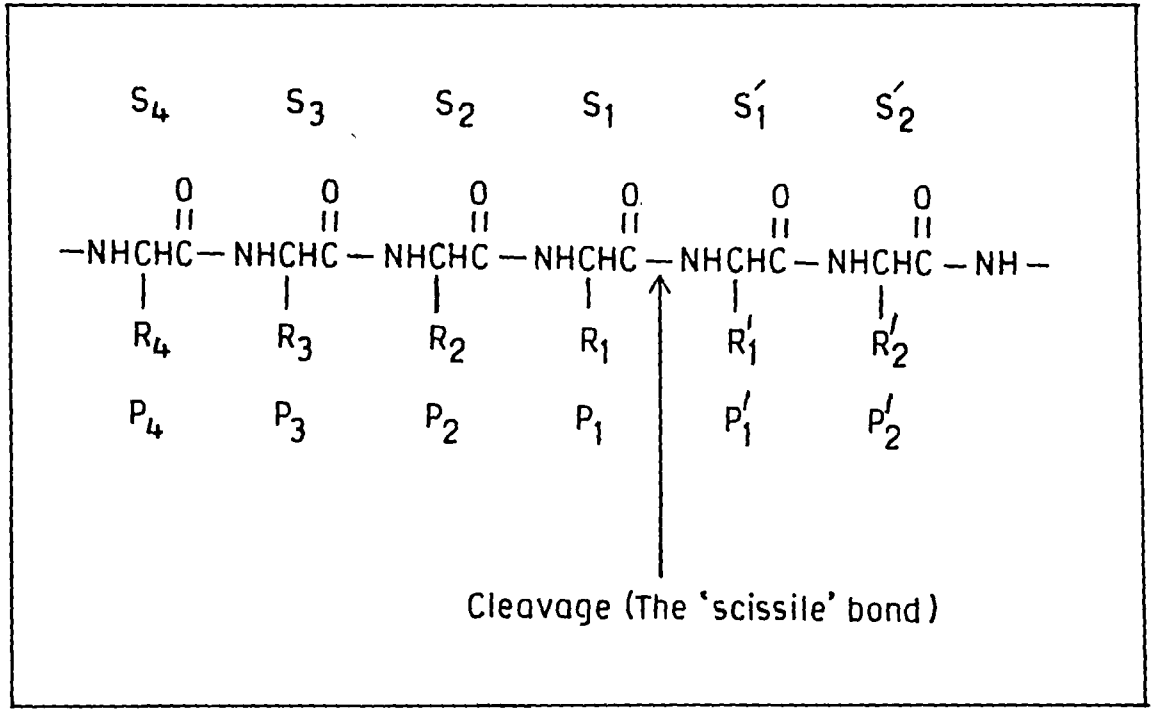
(B) Stability :

Cathepsin B is quite stable between pH 4.0-6.5 for long time, and appreciably near about the neutral pH zone. However, the enzymes is rapidly inactivated above the mild alkaline zone due to irreversible denaturation of the enzyme (Evans **et al.,** 1983; Bajkowski **et al.,** 1983;Khan **et al.,** 1986, Agarwal **et al.,** 1987b; Khouri **et al.,** 1991; Bromme **et al.,** 1993). The enzyme shows thermal stability below the physiological temperature (Khan **et al.,** 1986; Agarwal and Khan, 1987b; Turk **et al.,** 1993).

CATALYTIC SPECIFICITY :

The enzyme is active in the pH range of 4.0-6.8 and loses its activity irreversibly at mild and highly alkaline pH due to rapid denaturation (Mort **et al.,** 1980; Khouri **et el.,** 1991). **In vitro** studies of the specificity of cathepsin B have shown that the enzyme acts as a peptidyl dipeptidase with broad specificity on polypeptides such as glucagon (Aronson and Barrett, 1978), Fructose-1,6-diphosphate aldolase (Bond and Barrett, 1980) and

Fig. 2. Model for the binding sites for polypeptide substrates across the surface of the enzyme (Berger and Schechter, 1970)
The substrate residues are denoted by P (for peptide) and the enzyme subsites by S.


S4 S3 S2 S1 S1' S2'
—NHCHC— NHCHC — NHCHC — NHCHC — NHCHC — NHCHC — NH —
R4 R3 R2 R1 R1' R2'
P4 P3 P2 P1 P1' P2'
Cleavage (The 'scissile' bond)

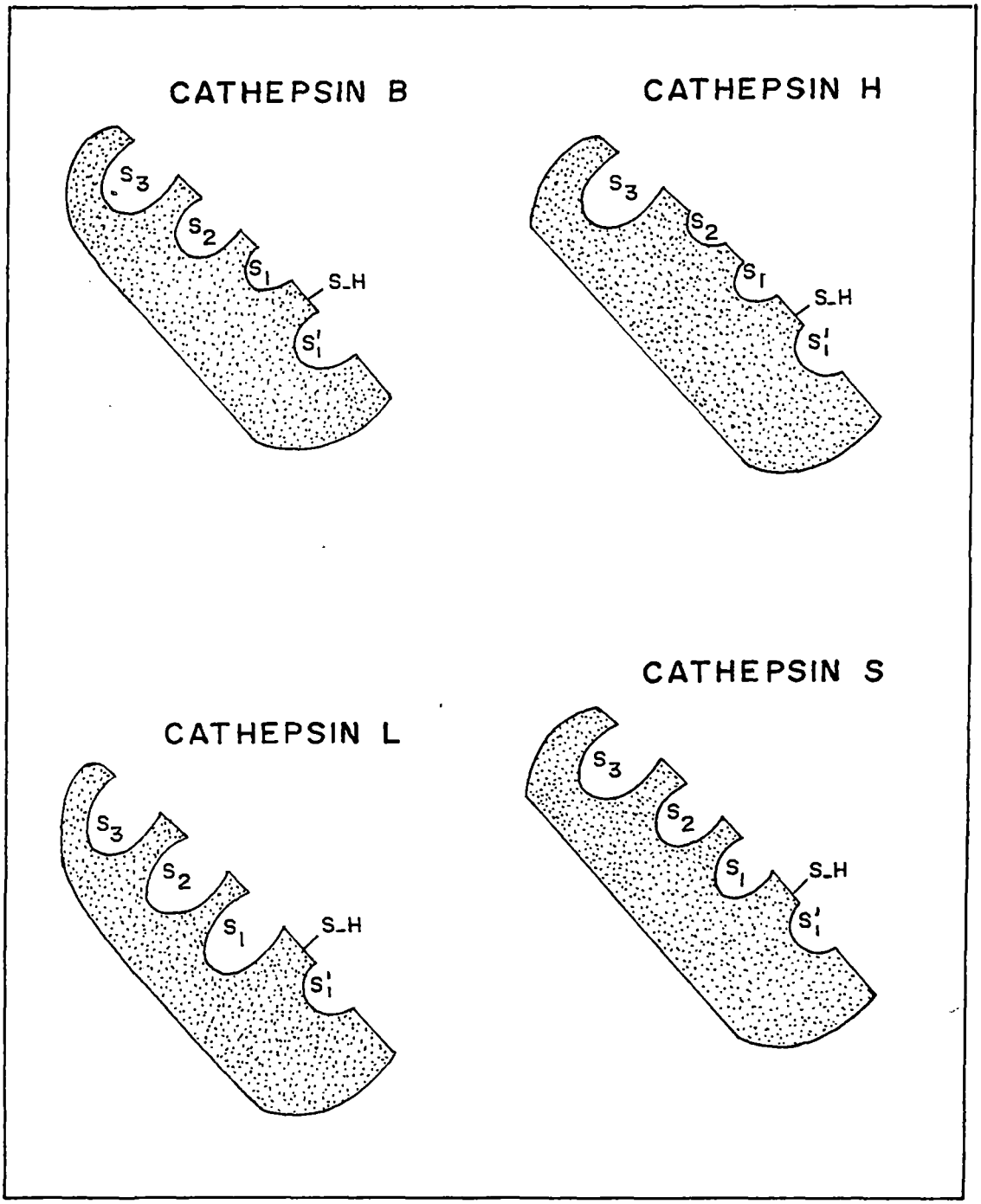
unspecifically on the insulin B chain (McKay **et al.,** 1983). On the other hand, synthetic substrates containing the -Arg-Arg-sequence (basic amino acid in P_1 and P_2, (Fig. 2) nomenclature as per Berger and Schechter, 1970) introduced by McDonald and Ellis, (1975) are extremely sensitive to cathepsin B showing trypsin-like specificity (Tchoupe **et al,** 1991; Xin **et al,** 1992).

MODEL OF ACTIVE SITE :

Depending upon the amino acid side chain they can accommodate in their specific pockets, cathepsin B, H, L and S can be distinguished from one another. The unique characteristics of cathepsin B, which distinguish it from other mammalian cysteine proteinases is that it can not readily accommodate aromatic side chain in S_1 (nomenclature according to Berger and Schechter 1970) and that it has peptidyl dipeptidase activity (Kirschke **et al.,** 1988). Khouri **et al.,** 1991; Hasnain **et al.,** 1992). Xin **et al.,** (1992) have demonstrated that cathepsin B does not bind rapidly to Z-leu-leu (I^{125}) Tyr-CHN_2 thereby confirming the restricted S_1 substrate binding pocket for cathepsin B. Cathepsin H on the other hand has restricted S_2 site and is thus in- capable of accommodating aromatic side chain in S_2 and also that this enzyme has aminopeptidase activity. Cathepsin L and S have similar extended S_1 and S_2 pockets, but differ significantly in affinities (Fig. 3).

Studies with a number of inhibitors like the analogues of E-64 show that cathepsin B can accommodate a bulky residue in a

Fig. 3. Model for the active-sites of cathepsin B, H, L and S (Xin **et al.**, 1992)


CATHEPSIN B
S3
S2
S1
S_H
S'1
CATHEPSIN H
S3
S2
S1
S_H
S'1
CATHEPSIN L
S3
S2
S1
S_H
S'1
CATHEPSIN S
S3
S2
S1
S_H
S'1

site near to the active site cysteine but close to that of S'_1 subsites (Murata **et al.,** 1991; Menard **et al.,** 1993). Thus the judicious use of inhibitors containing different combinations of amino acids at P_1, P_2, P_3 and P'_1 makes it easy to distinguish among these closely related cysteine proteinases. For example, inhibitors with aromatic side chain at P_1 would primarily block cathepsin S and L activity, while cathepsin B and H are less affected. On the contrary , presence of an aromatic amino acid at P_2 blocks the activity of all other cysteine proteinases but not that of cathepsin H (Baudys **et al.,** 1991; Ahmed **et al.,** 1992; Xin **et al.,** 1992; Bromme **et al.,** 1993; Giordano **et al.,** 1993)(Fig.3).

ACTIVATORS AND INHIBITORS :

ACTIVATORS :

Being a typical cysteine proteinase, cathepsin B requires the presence of sulfhydryl compounds to elicit its optimal activity at low pH. The most universally used sulfhydryl compounds include 2-mercaptoethylamine, cysteine, dithiothreitol, 2-mercaptoethanol and glutathione. The requirement of such compounds along with ethylenediamine tetraaceticacid (EDTA) is most probably to keep the active site cysteine (i.e cysteine-29 in cathepsin B) in the reduced atmosphere (Shutov **et al.,** 1987).

INHIBITORS :

A number of molecules can inactivate cysteine proteinases reversibly as well as irreversibly without significantly inhibiting other classes of proteinases. The reversible types of inhibi-

tors include low molecular weight mercurial compounds, D-Glucosamine-HCl and a wide variety of peptide 2-Aminoaldehyde derivatives like leupeptin, chymostatin, antipain, calpeptin, MDL etc. (Medhi 1991, Giordano **et al.,** 1990; Walker **et al.,** 1993; Montenez **et al.,** 1994).

The irreversible inhibitors specific for cysteine proteinases include several thiol blocking reagents such as iodoacetate, iodoacetamide, N-ethyl maleamide, p-chloromercurobenzoic acid, number of peptidyl diazomethyl ketones, fluoromethyl ketones, epoxysuccinyl peptides (oxiranes) like E-64 and their derivatives (Gour-Salin **et al.,** 1993).

Mounting numbers of such inhibitors and their derivatives have been synthesized in recent years and their specificity established. Among the cysteine proteinase inhibitors so far studied, N-(L-3-trans-propylcarbamoyloxirane-2-carboxyl)-L-isoleucine-L-proline or CA-074 and its methyl ester (CFA-074Me) proved to be the most specific and potent irreversible inactivators of cathepsin B (Oshita **et al.,** 1992; Sumiya **et al.,** 1992).

A similar derivative of E-64, N-(L-3-trans-ethocarboxyloxirane-2-carboxyl)-L-isoleucine-L-proline or CA-030 inhibits cathepsin B activity selectively **in vitro** but not **in vivo** as the ethyl-ester bond introduced to epoxysuccinyl group is hydrolysed by esterases (Towatari **et al.,** 1991; Oshita **et al.,** 1992; Giordano **et al.,** 1993).

Although the mode of action of these inhibitors are not clearly understood, yet it is certain that they inactivate the

active site thiol (cysteine-29) by alkylating it. All the inhibitors show significant inhibition at low pH since the small molecular weight thiols are in protonated forms under such conditions (Angliker **et al.,** 1991).

Leupeptin and its analogue have been found to inactivate cathepsin B and related proteinases efficiently due to the nucleophilic attack on the active site thiol at the aldehyde carbonyl of the bound inhibitor to form reversibly a hemithioacetal. In case of inactivation by E-64 and its analogue, the formation of strong C-S bond between the oxirane C-2 of E-64 (or its analogue) and the active site cysteine is not easily reversible by attack of water or other nucleophiles to reactivate the enzyme, and thus, renders it irreversibly inactivate (Medhi, 1991; Giordano **et al.,** 1993).

Besides these low molecular weight inhibitors, many cysteine proteinases are presumably regulated by naturally occurring inhibitory proteins, known as cysteine proteinase inhibitors (CPIs) or thiol proteinase inhibitors (TPIs) found primarily in the cytoplasm, extracellular fluids and also in association with lysosomal membranes (Lah **et al.,** 1992).

The most prominent cysteine proteinase inhibitors belong to the cystatins, a superfamily of evolutionarily related proteins. Cystatins are classified into three families, namely, Cystatin I (or stefins), Cystatin II (or cystatins) and Cystatin III (or Kininogens). This classification is based in respect to their molecular size, presence of carbohydrate moieties, number of

disulfide bonds, primary structure and subcellular localization (Barrett **et al.,** 1986).

Members of family II cystatins which include cystatin C, S, SN, SA etc. besides a few others occur both intracellularly and extracellularly in various tissue fluids and display a very high affinity for cathepsin B in comparison to other cysteine proteinases (Cortichicto **et al.,** 1992; Barka **et al.,** 1992). Lenney **et al.,** 1982) had earlier demonstrated the presence of a number of high molecular weight CPIs from serum which were identified as haptoglobin, α_2 macroglobulin, α and α_2 CPI. Recently it has been suggested by Hiwasa **et al.,** (1987, 1993), that the ras- oncogene products resemble low molecular weight cystatins and act as potent inhibitors of cysteine proteinase especially cathepsin B and L.

STRUCTURAL STUDIES :

Cathepsin B is roughly disc shaped with a diameter of 50 A° and thickness of 30 A°. Like papain, cathepsin B has two domains. One consisting of residues 12-134, 153-158 and 250-252 of the single chain polypeptide forms the L- domain while, the rest of the residues form the R-domain. The catalytic centre with cysteine-29 (corresponding to cysteine-25 in papain) is located at the cleft between these two domains (Musil **et al.,** 1991, Sumiya **et al.,** 1992, Yamamoto **et al.,** 1992).

Although cathepsin B shows great sequence homology with cathepsin H, L, S and papain, characteristic differences are observed between them especially corresponding to the tertiary structures around the active centre. The S site of cathepsin B

consisting of neutral and hydrophobic environment is spatially smaller and shallower than that of papain, while , the S' subsite of the former is comparatively wider than that of the latter. The side chain of catalytic His-197 (or His-199 in two chain form) of cathepsin B is always preceded by two glycine residues: Gly-195 and Gly-196 (or Gly-197 and Gly-198) as against the consensus Asp-158 preceding the corresponding catalytic His-159 in papain. This, therefore provides a flexible S_2 site for cathepsin B, as against papain.

Presence of Glu-247 (Glu-245) in cathepsin B has been linked to P_2 specificity for arginine as against corresponding Ser-205 in papain. However, the major structural differences between cathepsin B and other members of papain groups eg. papain actinidin, cathepsins H, L and S is the strategic presence of His-108 and His-109 (or His-110 and His-111) in the S'subsite of cathepsin B. These two histidine residues do not have any equivalence in other members of papain groups described above. Hence, dipeptidyl carboxypeptidase is the unique characteristics of cathepsin B (Baudys **et al.,** 1991; Musil **et al.,** 1991; Sumiya **et al.,** 1992; Buttle **et al.,** 1992; Hasnain **et al.,** 1993). Recent studies showed the presence of 16 cysteine residues in bovine spleen cathepsin B, of which 14 are involved in the formation of 7 disulfide bridges and two are free (Baudys **et al.,** 1991).

GENE STRUCTURE :

Although the primary structure and partial cDNA sequence of cathepsin B from various sources had been worked out long time

back, the complete structure of cathepsin B gene was not known until recently when the mouse cathepsin B gene structure first came in (Qian **et al.,** 1990).

Mouse cathepsin B gene is about 20 bp long and contains 9 introns and 10 exons that transcribes about 2.2 kb mRNA with a coding region for 339 residues long proenzyme. No introns have been found at the junction of signal peptide, pro-peptide, mature enzyme, carboxy-terminal extension or at the cleavage site between light and heavy chain. On the contrary, the highly conserved sequence around the active site cysteine (Cys-29) is found to be split by an intron. In contrast to cathepsin B gene, cathepsins H and L genes comprise 12 and 8 exons that span over 21.5 kbp and 8.5 kbp respectively. Cathepsin B gene lacked TATA box like cathepsin H, and L, but, contained comparatively higher G/C rich exon-1, in comparison to the latter two (Qian **et al.,** 1991; Ishidoh **et al.,** 1991; Chouhan **et al.,** 1993). Heterogeneities of mRNA for cathepsin B have been reported from almost all cancer and malignant cells. The presence of 4.0 and 5.0 kb transcripts in addition to normal 2.2 kb for cathepsin B had raised the suspicion about the possible chromosomal rearrangement or presence and expression of separate genes. However, sequence studies of the 4.0 and 5.0 kb transcripts showed the presence of usually long extended 3' untranslated regions, but the coding regions are well conserved as in 2.2 kb transcript, with similar translated products. Thus the possibility of alternate gene splicing or expression of two separate genes is ruled out.

Hence, the only probable heterogeneity in cathepsin B population must arise due to post translational modifications (Qian **et al.**, 1991; Mach **et al.**, 1992; Fox **et al.**, 1992; Mordier **et al.**, 1993).

Recently, Fong and his co-workers (1992) have confirmed the mapping of human cathepsin B gene to chromosome 8, while cathepsin L and cathepsin H genes have been assigned to chromosome 9 and 15 respectively.

PHYSILOGICAL SIGNIFICANCE :

Cathepsin B, besides a few other lysosomal cysteine proteinases (eg. cathepsin L, H, and S) has been implicated in a varieties of physiological and pathological processes (Katunuma **et al.**, 1988). Under normal conditions the enzyme participates in cell differentiation (Kirschke **et al.**, 1983), degradation of extracellular proteins taken into cells by endocytosis (Huisman **et al.**, 1974), the turnover of intracellular proteins (Kar and Pearson, 1977; Shaw and Dean, 1980; Sohar and Katona, 1992), cell invasiveness (Mullins **et al.**, 1983; Young and Spevacek, 1993), processing of hormones (Quinn and Judah, 1978; Dockerty **et al.**, 1984), post ribosomal processing of proteins (Mizuno **et al.**, 1982), antigen presentation (Coetzer **et al.**, 1991;Matsunaga **et al.**,1993) and platelet aggregation (Sloane **et al.**, 1992).

However, in cases where the enzyme becomes deregulated, cathepsin B has been implicated in a variety of diseased states. Several diseases that involve aberrant protein turnover such as muscular dystrophy (Katunuma **et al.**, 1986; Matsuishi **et al.**,

1992; Golde **et al.**, 1992; Takeda **et al.**, 1992 ; Belkhou **et al.**, 1994), bone resorption (Delaisse **et al.**, 1984; Sasaki **et al.**, 1992), myocardial infarction (Bolli **et al.**, 1983; Laszlo **et al.**, 1992), rheumatoid arthritis (Trabandt **et al.**, 1991), inflammation and pulmonary emphysema (Harris **et al.**, 1975), tumour growth (Sedo **et al.**, 1991; Hirano **et al.**, 1993), malignancy (Sloane **et al.**, 1992; Liu **et al.**, 1992; Gabrijelcic **et al.**, 1992; Lah **et al.**, 1992; Schultz **et al.**, 1994), increased glomerular basement membrane permeability and resultant proteinuria and renal disorder (Baricos **et al.**, 1990) are among the few important pathological disorders which are directly or indirectly correlated with over expression of cathepsin B.

Involvement of cathepsin B in above mentioned physiological and pathological processes have, however been questioned due to its low stability and minimal catalytic activity at physiological pH, temperature and ionic strength. Moreover, the data available on differential species and tissue specificities of cathepsin B are also fragmentary. A thorough investigation is therefore needed before a definite comment on the role of cathepsin B **in vivo** can be made.

In an attempt to further add to the existing knowledge on cathepsin B from different sources, we have undertaken this study involving purification and physico-chemical characterization of the enzyme from a hitherto unstudied buffalo kidney source. The data has been compared with similar information available for cathepsin B from other sources.

EXPERIMENTAL PROCEDURES

A. Materials :

Proteins :

Cathepsin B was isolated from buffalo kidneys collected from the local abattoir. Bovine serum albumin (lot no 86F-0714), Ovalbumin (lot No. 23F-8175), chymotrypsinogen-A (lot No. 29C-8010), Carbonic anhydrase (lot No. 115F-94101), cytochromme C (lot No. 124F-7155), Myoglobin (lot No. 61F-7035), ribonuclease A (lot No. 84F-8145), carboxypeptidase A (lot No. 20H-800), rabbit muscle aldolase (lot No. 63H 9514) were procured from Sigma Chemical Company, U.S.A. Bovine milk casein was obtained from Sisco Res. Lab., India.

Reagents used in end group analyses :

Dansyl chloride (lot No. 36F-0207), dansyl derivatives of alanine, arginine, asparagine, aspartic acid, glutamic acid, glutamine, glycine, isoleucine, leucine, lysine, methionine, phenylalanine, proline, serine, threonine, tryptophan, tyrosine and valine and iodoacetamide (lot No. 60F-5033), standard amino acid kit (lot No. 45F-9004) were purchased from Sigma Chemical Company, U.S.A. Micropolyamide 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 :

Gel chromatography media such as Sephadex (G-25, G-75 and G-100), Blue dextran 2000, ion exchange media like Diethyl amino-ethyl (DEAE) Sephadex A-50 and Carboxymethyl (CM) Spehadex C-50,were obtained from Pharmacia Fine Chemicals, Uppsala, Sweden. chromatofocusing media viz., Polybuffer exchanger (PBE-94) and polybuffer 74, amberlite MB-3 (lot no. 81F-0311) were obtained from Sigma Chemical Company, U.S.A.

Reagents used for polyacrylamide gel electrophoresis :

Reagents used for polyacrylamide gel electrophoresis in the presence and absence of sodium dodecyl sulfate (SDS) with the names of their suppliers in parentheses were : acrylamide, N,N'methylene bis acrylamide, N,N,N',N'-tetramethylethyenedia-mine, 2-mercaptoethanol, coomassie brilliant blue R-250, gly-cine, Tris (Hydroxymethyl aminomethane), sodium dodecyl sulfate (Sigma Chemical Company, U.S.A.), ammonium persulfate, sucrose and glycerol (E.Merck India), amidoschwarz and bromophenol blue (BDH, England).

Other reagents :

Acetic anhydride, N-acetyl-L-cysteine, Arg-BNA, BANA, BAPNA, DMSO, Leu-BNA, 2-napthylamine, p-nitroaniline, DTNB, FCA, IFA, antipain, chymostatin, E-64, leupeptin, pepstatin A, cys-teine base, cysteamine-HCl, dithiothreitol, glutathione, thiogly-cerol, iodoacetic acid, p-mercuric benzoic acid, guanidine hydro chloride, sodium azide, urea and calibration mixture for amino

acid analysis were obtained from Sigma Chemical Company, U.S.A. Coomassie brilliant blue G-250, Brij 35, o-pthalaldehyde were the products of Serva Biochemicals, U.S.A.. Alhydrogel was obtained from Superfos Speciality Chemicals a/s, Denmark. Arg-MCA, Z-Arg-Arg-MCA, Z-Phe-Arg-MCA, and MCA, were the products of Peptide Institute, Inc., Japan. Ammonium sulfate, TCA, chromic acid, N-1-napthylethylenediamine-HCl and standard buffer tablets of different pHs were purchased from BDH, England. Agarose, Ammonium sulfamate, EDTA, histidine, papain, methyl cellusolve, ninhydrin were from Sisco Res. Lab. India. D-Glucose, ascorbic acid, potassium ferricyanide and orthophosphoric acid were purchased from Glaxo Lab., Bombay, India. Phenol, perchloric acid, sodium hypochlorite, hydrogen peroxide, potassium sulfate, ethanol , N-caprylic acid were the products of Fluka chemical AG, Switzerland. All other reagents were either analytical grade or the best commercially available.

Miscellaneous :

Dialyser tubings of different diameters were purchased from Sigma Chemical Company, U.S.A. Millipore filters (pore size 0.22 μM, 0.45μM), 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 supplied by a local supplier from Guwahati, India.

Either glass double distilled or filtered Milli-Q-deionized water (Millipore Corporation, Belford, U.S.A.) was used throughout these studies.

B. METHODS :

1. pH measurements :

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

2. Optical measurements :

a. Measurements of Optical Density:

Light absorption measurements in the ultraviolet as well as visible region were performed on either Backman UV-Visible spectrophotometer, model-26, or Jasco UVIDEC-610 Double Beam, spectrophotometer using quartz (in UV region) or glass (for visible region) cuvettes of 1cm path length. All measurements were done at room temperature, unless stated otherwise.

b. Fluorescence measurements :

Fluorometric studies were done on a Shimadzu RF 540 spectroflurophotometer, fitted with a thermostat. Fused quartz cuvettes of 1 cm path lengths were used for all the experiments.

3. Centrifugation :

Centrifugation was carried out at 4°C in Backman Refrigerated Centrifuge Model J 2-21. Microcentrifugation were done in Appendorf microcentrifuge.

4. Lyophilization :

Lyophilization of various samples were done in LSL Secfroid, Lyolab B II operated between -32 to -38°C.

5. Determination of protein concentration :

Protein concentration of solutions were determined either by the dye-binding method of Bradford (1976), using bovine serum albumin (BSA) as standard, or directly by measuring their absorbance at 280 nm using values of their specific extinction coefficient.

a. Dye-binding method :

The method originally developed by Bradford (1976) and modified by Bio-Rad Ltd., USA (1979), consists of the following set ups:

Preparation of colour reagent :

One hundred milligrams of coomassie brilliant blue G-250 was dissolved completely in 50 ml of ethanol (95%) and to it 100 ml of 85% (w/v) orthophosphoric acid was added. The contents was thoroughly mixed and transferred to a dark bottle with a tight stopper. This was the Bradford stock reagent which was kept refrigerated for long term use.

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

Assay of protein :

To 1.0 ml of protein solution. 5.0 ml of the Bradford working solution was added and mixed with the care that no froathing should occur. Colour was allowed to develop for 10-15 minutes at room temperature and the intensity of colour was determined at 590 nm within 30 minutes against a suitable reagent blank. Protein concentration was determined with the help of a standard curve prepared as above with varying concentrations of BSA.

b. Spectrophotometric method :

The Optical Density (OD) of different protein solutions measured at 280 nm was divided by their respective specific extinction coefficient ($E^{1\%}_{1cm}$). The value of the quotient gave the amount of protein in grams per 100 ml of the solution. Correction for the 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.

6. Chromatography :

a. Gel filtration :

Gel filtration chromatography were done on columns packed with Sephadex G-50, G-75 and G-100 during the various steps of purification and characterization of the enzyme. Analytical gel chromatography was performed on Sephadex G-100 column for the determination of hydrodynamic parameters.

Gel were prepared by swelling fresh and dry powder of Sephadex in excess of distilled water at 40-50°C for 10-24 hrs as recommended by the manufacturer. The fine particles of the gel slurry were removed by decantation or by gentle suction. The process was repeated until all the fines were completely removed from the gel slurry. The gel slurry with about 75% of settled gel was degassed well under vacuum to remove the trapped air and brought to the room temperature before packing it into the chromatographic column. Clean glass was mounted vertically and filled with equilibrating buffer up to one-fifth of the column height. The free end of the outlet tubing was positioned 5cm below the top of the column. 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 half an hour and then constant hydrostatic pressure was applied through a peristaltic pump to settle the gels at flow rate of about 15 cm/hr.

For equilibrating the column, a volume of buffer equal to 3 times the total bed volume was passed through the column at a constant flow of 25 ml/hr. Homogeneity of the packed gel bed was checked by monitoring the progress of a narrow band of Blue Dextran 2000 and potassium ferricyanide ($K_3(FeCN)_6$) solution (2mg/ml). The elution volume 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

2mm of buffer above the gel. 2-5 ml of sample containing 10-100 mg of protein was then carefully applied on the column with the help of a thin tubing and allowed it 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 of appropriate sizes (3-5 ml) were collected with the help of automatic fraction collector and monitored spectrophotometrically.

b. Thin layer chromatography :

Thin layer chromatography was performed on micropolyamide sheets of 5x5 cm (Pierce Chem. Co.). Samples were applied above 5mm from the bottom with the help of fine capillary tubes. This process was repeated until sufficient amount of the sample was spotted and dried with the help of a hair dryer. Ascending chromatography was then carried on in 150 ml chromatographic chamber containing the appropriate solvent system. After the completion of the run the sheets were removed and dried. The chromatograms were detected as fluorescent spots while viewed under the ultraviolet lamp and the R_f values were computed by dividing the distance moved by the samples with that of the solvent front.

7. Gel Electrophoresis :

Electrophoresis in polyacrylamide gels were done in absence and presence of detergents using the methods of Davis (1964) and

modified technique of Laemmli (1970) respectively.

a. Non-denaturing polycrylamide gel electrophoresis (PAGE) :

Clean gel columns (0.5x10 cm) were marked up to 9.0cm and placed vertically on a stand after sealing the lower end. 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 TEMED) and 1 volume of freshly prepared ammonium persulfate solution (2.7 mg/ml). The resulting solution thus formed 7.5% gel. 50-100 µg of protein was carefully applied on the layer of the gel after mixing with glycerol and 0.1% bromophenol blue. The electrophoresis was carried out with anodic current of 2 mA/tube for 2-3 hours using Tris-Glycine buffer, pH 8.3 (prepared by mixing 2.88 gms glycine and 0.6 gms Tris/l distilled water). The current was stopped when the bromophenol marker reached almost near to the bottom of the tube. The gels were removed by flushing in distilled water through the inner wall of the tube with the help of a syringe and needle. Gels were stained with 0.01% amido black solution for 20 mins and finally destained by a destaining solution containing 7.0% acetic acid in 5% methanol.

b. Sodium dodecyl sulfate-polyacrylamide gel electrophoresis :

Polyacrylamide gel electrophoresis of the protein in presence of 1.5% sodium dodecyl sulfate was done both in presence and absence of 0.1% 2-mercaptoethanol. A 10% gel was prepared by mixing 10.3 ml water, 8.25 ml solution A (30% Acrylamide, 0.8%

N,N,bisacrylamide), 6.25 ml solution B (18.7 gms Tris + 2.0 ml 20% (w/v) SDS made up the volume to 100 ml after carefully adjusting the pH to 8.8 with 6.0 N HCl), 0.375 ml freshly prepared ammonium persulfate solution (20 mg/ml) and 0.025 ml TEMED.

Protein samples were denatured in 1.0% SDS by heating in a boiling water bath for 10 mins followed by overnight incubation with 0.1% 2-mercaptoethanol (if required). Electrophoresis of the denatured protein samples was 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 as the running buffers. Before loading the samples in the gel tubes, pre-run was done for 30 mins at a constant current of 2.5 mA/tube. Prior to applying, the samples were mixed with 20% glycerol and 0.1% bromophenol blue. 50-100 µg of protein per tube was applied on the gel surface and electrophoresis was allowed to proceed at a constant current of 3 mA/tube until the band of bromophenol blue reached nearly to the bottom of the gel. The gels were removed as described previously and finally stained with coomassie Brilliant Blue R-250, and destained in a solution containing 7% acetic acid in 5% methanol. The relative mobilities (R_m) of electrophoresed protein samples were determined by the standard procedure of Laemmli (1970), using the expression:

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

8. Isolation and purification of cathepsin B :

Cathepsin B was isolated from buffalo kidney according to the method of Takahashi **et al.,** (1984a) and Ahmad **et al.,**(1989), incorporating suitable modifications. The procedure is briefly described as follows:

a. Homogenization :

Buffalo kidneys weighting 400 to 600 gm from freshly slaughtered buffalo were collected over ice from local abattoir and kept frozen at -20°C until use. The frozen kidneys were thawed at room temperature and washed with excessive amount of distilled water followed by 1mM EDTA solution. The soft mass of tissues weighting 400 gm (obtained through 600 gm of kidneys after removing peripheral membranes, fats and connective tissues) was homogenized with 200 ml of 3% NaCl solution containing 1mM EDTA and 15 mM HCl (pH 1.8). This was kept under continuous stirring for 6 hrs at 4°C.

b. Acid extraction :

The pH of the homogenate thus obtained was adjusted to 3.8 by gradual addition of chilled HCl (2 N) and left for continuous stirring at 4°C for another 6-8 hours. The content was then centrifuged twice at 25,000 g for 20 mins each and the clear supernatant was collected for the next step.

c. Ammonium Sulfate fractionation :

The clear supernatant obtained after acid extraction was subjected to salt fractionation. The protein fraction precipitat-

ing between 40-75% ammonium sulfate saturation was collected and dissolved in minimum amount of chilled distilled water. This protein solutions was then excessively dialyzed against chilled distilled water followed by 50 mM sodium acetate buffer, pH 5.0 containing 1mM EDTA and 0.02% sodium azide. The whole content was once again centrifuged at 25,000 g for 20 mins and the clear supernatant thus obtained was collected for further purifications.

d. Gel- Chromatography on Sephadex G-75 :

Protein solution obtained through salt fractionation, described as above was concentrated and chromotographed on a Sephedex 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 at 280 nm for protein concentration and was also subjected to enzymatic assay using BANA as the substrate. The enzymatically active fractions were pooled and concentrated for use in the next step.

e. Ion-exchange chromatography on CM-Sephadex column :

The enzymatically active fractions obtained through gel filtration chromotography was excessively dialysed against 20 mM. sodium acetate buffer, pH 4.8 containing 1 mM EDTA and 1.4 mM 2-mercaptoethanol and applied on a CM-Sephadex-C-50 column (1.6x12 cm), pre-equilibrated with the above buffer. The column was developed with the same buffer at pH 4.8 followed by the stepwise

elution of bound protein fractions with the above buffer, at pH 5.6, 6.0 and finally using the sodium chloride gradient (0-1.0 M) at pH 6.0. Protein estimation and enzymatic assay of the eluted fractions were measured spectrophotometrically and spectrofluro-metrically. Protein fractions eluted at pH 5.6 were collected, pooled and processed for the next step.

f. Chromatofocusing on PBE-94 followed by rechromatography on Sephadex G-100 Column :

Protein fractions eluted with 20 mM sodium acetate buffer pH 5.6 containing 1 mM EDTA and 1.4 mM 2-mercaptoethanol were collected, pooled and dialysed excessively against 25 mM histis-dine buffer, pH 6.3, containing 1 mM EDTA and 1.4 mM 2-mercapto-ethanol. This was finally applied on a PBE-94 column (0.9x12 cm), pre-equilibrated with the same histidine buffer and eluted with decreasing pH gradient by applying diluted polybuffer 74-HCl, pH 4.0 (as suggested by the manufacturer). Protein estimation of the eluted fractions were made spectrophotometrically at 280 nm followed by their enzymatic assay. For removal of polybuffer from the eluted protein, the enzymatically active fractions obtained as above were pooled and subjected to salt fractionation with ammonium sulfate (80% saturation). The protein fraction thus precipitated was excessively dialysed against distilled water and subjected to gel filtration chromatography on a Sephadex G-100 column (1.6x92 cm) pre-equilibrated with 60 mM sodium phosphate buffer, pH 6.0, containing 1 mM EDTA and 0.02% sodium azide. The

enzymatically active fractions thus obtained were pooled, concentrated and stored at -20°C until further use.

9. CHEMICAL ANALYSES :

a. Chemical modification of cathepsin B by performic acid oxidation :

Performic acid oxidation of cathepsin B was done according to the method described by Hirs (1956). Briefly, 10-15 mg of halide free cathepsin B was dissolved in 2.5 ml of 99% formic acid in a flask and 0.5 ml of anhydrous methanol was added to it with constant stirring. In another flask performic acid was prepared by adding 5 ml of 30% hydrogen peroxide (H_2O_2) to 95 ml of 99% formic acid. The mixture was allowed to stand at room temperature for 2 hrs. The contents of the two flasks were then cooled to -10°C and mixed together. The reaction was allowed to proceed at -10°C for at least 2.5 hrs. The total content was diluted with equal volume of distilled water and lyophilized. The lyophilized protein was washed twice with distilled water and lyophilized again so as to remove traces of performic acid. Alternatively, the performic acid oxidised cathepsin B was recovered through the solution by precipitation with tri-chloroacetic acid (TCA) to a final concentration of 10%. The precipitated protein was repeatedly washed with 10% TCA until it gave a negative test for peroxide with KI-starch paper. The protein was further washed with sufficient volume of absolute ethanol followed by two washings with ether.

b. End group analyses :

(i). Identification of NH_2-terminal amino acid residue :

Determination of NH_2-terminal amino acid residue of the purified cathepsin B from buffalo kidney was done according to the method of Gray (1967). To 1 ml of protein solution containing 10 n mole (0.026 mg) of performic acid oxidised cathepsin B, were added urea and sodium bicarbonate to a final concentration of 8 M and 0.5 M, respectively and left at 37°C for 1 hr. To this protein solution was added an equal volume of dansyl chloride (20 mg/ml) solution in acetone and incubated at 37°C for about 18 hrs. The whole content was then dialyzed excessively against distilled water to remove free dansyl chloride, salts, and other low molecular weight substances. The dialyzed protein was then transferred into a hydrolyzing tube (Pierce Chem. Co.) and dried under vacuum. Finally, 0.5 ml of 5.7 N freshly distilled HCl was added to the protein and the tube was closed air tight. Hydrolysis was performed in thermo-recti (Pierce Chem. Co.) at 110°C for about 18 hrs. The content was evaporated to dryness and the residue was dissolved in 50% pyridine aqueous solution. Identification of the dansylated amino acid was finally done with thin layer chromatography (TLC) on polyamide sheets.

(ii). Identification of COOH-terminal amino acid residue :

Determination of COOH-terminal amino acid of buffalo kidney cathepsin B was done according to the method of Narita (1970) as described below :

To 4 ml of performic acid oxidised cathepsin B (0.4 mg) solution in 60 mM sodium phosphate buffer, pH 8.0, were added 1.8 gms urea, 50 µl 2-mercaptoethanol and 46.23 mg iodoacetamide to give final concentrations of urea, 2-mercaptoethanol and iodoacetamide as 6.0 M, 1% and 0.05 M respectively. The reaction mixture was incubated at 37°C for overnight followed by excessive 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 IU) of the diisopropyl phosphorofluoridate (DFP) treated enzyme in 5.0 ml of water. The suspension was centrifuged and the supernatant thus obtained was discarded. The residue was collected and placed on an ice bath for 5-6 mins prior to addition of 0.1 ml sodium bicarbonate (1%). Next, 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 with 0.1 N HCl to 8.0. The concentration of the enzyme was determined spectrophotometrically using the extinction coefficient of 8.6×10^4 /cm/M (Neurath, 1955). The molecular weight of the enzyme was taken to be 34,000 (Narita, 1970). The enzyme solution was diluted to a concentration of 1 mg/ ml with 60 mM sodium phosphate buffer pH 8.0 containing 6.0 M urea.

The enzymatic reaction between carboxypeptidase A and denatured cathepsin B was performed in the molar ratio of 2:1 and 5:1 at room temperature. One ml aliquotes were withdrawn from the reaction mixture at various time intervals (eg., 0, 5, 10, 20,

30, 60 and 120 mins) and the reaction was stopped 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 the addition of the substrate (cathepsin B). The acid precipitated proteins were removed by centrifugation and the supernatant thus formed was analysed for the liberated amino acid(s) by running TLC after dansylation.

c. Determination of free thiol group(s) :

The free sulfhydryl content of the purified but unmodified cathepsin B, both under native as well as denatured conditions (8.0 M urea) was determined according to the procedure described by Ellman (1959).

Ellman's reagent was prepared by dissolving 10 mM 5,5'-dithiobis-(2-nitrobenzoic acid) (DTNB) in phosphate buffer (0.1 M), 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 0.2 ml enzyme and 2.8 ml of phosphate buffer described above without urea or with 8.0 M urea) and the optical absorbance at 412 nm was continuously measured for about 30 mins until no further increase was observed. The sulfhydryl content of the protein was calculated by using the equation:

$$C = AxD/E$$

where, C was the concentration of thiol group(s), A was the absorbance at 412 nm, E was the extinction coefficient of the

coloured complex (13,600/cm/M) and D was the dilution factor. The free -SH group (s)/protein (mole /mole) was obtained from the ratio of the total sulfhydryl and protein contents.

d. Determination of tryptophan residues :

Total tryptophan residues of unmodified cathepsin B was determined colourimetrically by the method of Spies and Chambers (1949) using *p-Dimethylaminobenzaldehyde (DAB)*.

To 0.5 ml of salt-free enzyme solution (containing known amount of protein), freshly flushed with nitrogen, was added 4.0 ml of (23.8 N) H_2SO_4 and mixed thoroughly. 0.5 ml of freshly prepared DAB solution (30 mg/ml in 2.0 N sulphuric acid) was then added to it and mixed well. The reaction mixture was incubated for overnight (about 16 hrs) at room temperature in dark. Following this, 0.1 ml of freshly prepared aqueous solution of $NaNO_2$ (0.045%) was added to the reaction mixture and mixed properly. After standing further for 60 mins 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 similar way with standard tryptophan solution.

e. Amino acid analyses :

Amino acid composition of the purified cathepsin B was done by using High Performance Liquid Chromatography (HPLC) (Shimadzu, Model LC-4A) using single Na^+ type cation exchange column (ISC-07/S1504 Na^+ from Shimadzu Co.). The following solutions were used:

I. Sample buffer	:	0.2 M sodium citrate buffer pH 2.2, containing 7% (v/v) ethanol). The pH adjustment was done with 60% 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 (0.216 M) and potassium sulfate (0.108 M) in distilled water.
VI. OPA solution	:	200 mg o-pthalaldehyde (OPA) 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, to the final volume of 250 ml with distilled water.
VII. Sodium Hypochlorite: solution		50 µl sodium hypochlorite/250 ml reaction reagent.

Preparation of sample for HPLC analysis :

0.5 ml aliquotes of protein solution (containing about 5.0 n mole of purified cathepsin B) were taken in a set of hydrolysis tubes (Pierce Chem. Co.) and dried under vacuum. 0.5 ml of freshly distilled 5.7 N HCl was then added to it and sealed properly. Hydrolysis was performed at 110°C for 6, 12, 18, and 24 hours after which the HCl was evaporated by passing a stream of nitrogen through the protein-HCl solution. After all the HCl was evaporated, 100 µl of HPLC sample buffer, pH 2.2 (described above) was added to the hydrolysate, centrifuged and finally

filtered through millipore filter (0.45 μM) before injecting into the HPLC column. The procedure was same for both modified as well as unmodified, but, denatured cathepsin B.

f. Estimation of carbohydrates :

Total carbohydrate content of the enzyme was estimated by phenol-sulphuric acid method of DuBois **et al.,** (1956), using D-glucose as standard.

1.0 ml of the enzyme solution (containing varying amount of cathepsin B), was mixed with 1.0 ml of 5% (w/v) phenol solution and the mixture was left for 2 mins. 5.0 ml of concentrated sulphuric acid was then carefully added to the mixture, mixed well and left for development of colour at room temperature for 30 mins. The intensity of the brown colour thus formed was measured at 485 nm against a suitable reagent blank. Standard curve was prepared in the same way with varying concentrations of D-glucose.

10. KINETIC STUDIES :

a. Enzymatic assay with synthetic substrates :

(i). Fluorimetric assay with BANA hydrolase activity :

Enzymatic assay of cathepsin B against α-N-benzoyl-DL-arginine -2-napthylamide as the substrate was performed according to method described by Khan **et al.,** (1986). Briefly, 0.1 ml of enzyme solution was incubated with 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) for 30 mins at 37°C. The reaction was

initiated by addition of 1.0 ml of the substrate solution (prepared by dissolving 10 mg BANA in 0.3 ml of DMSO followed by dilution to desired concentration with activator buffer, described above). BANA hydrolase activity was measured fluorimetrically by monitoring the release of 2-napthylamine continuously for 30 mins at 37°C, using the excitation and emission wavelength of 335 and 410 nm respectively . The amount of 2-napthylamine thus released was calculated using a standard curve prepared with 2-napthylamine in the same manner, as described by Barrett and Kirschke (1981). One unit of the enzyme activity was defined as the amount of enzyme required to release 1 μMol of 2-napthylamine per hour at 37°C.

(ii). Spectrophotometrical assay of BANA hydrolase activity :

Method of Martineck **et al.,** (1964) with slight modification was used for spectrophotometric assay of BANA hydrolase activity of the enzyme. Briefly, 1.0 ml of enzyme solution in activator buffer was incubated for 30 mins at 37°C. 0.5 ml of this preactivated enzyme solution was pipetted out in a separate test tube for preparing control. To the remaining 0.5 ml enzyme solution, 0.5 ml of substrate solution (0.1% BANA in 3% DMSO, prepared as above) was added, mixed by inversion and incubated at 37°C for 30 mins or 1 hr. The reaction was finally stopped by the addition of 0.5 ml of 4.0 N HCl and the amount of 2-napthylamine thus formed was determined by diazotization method as described below.

The reaction mixture (1.5 ml) was mixed with 0.5 ml sodium nitrite (0.2% w/v) and left for 3 mins. Following this, 1.0 ml of

ammonium sulfamate solution (0.5% w/v) was added and mixed properly . After another 3 mins of standing at room temperature, 2.0 ml of dye solution (N-1-napthylethylenediamine-dihydrochloride (0.05% w/v in absolute alcohol) was added to it. The colour was allowed to develop at least for 1 hr. and the intensity of the blue colour thus formed was measured at 540 nm against suitable enzyme blank. The blank was prepared in the same way, only difference being that the HCl was added to the enzyme before addition of the substrate.

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

(iii). Spectrophotometric assay of BAPNA hydrolase activity :

To 0.5 ml of preactivated enzyme solution was added 0.5ml of BAPNA solution (0.1% prepared in the same way like BANA). The reaction was allowed to proceed at 37°C for 30 mins or 1 hr as required and finally terminated by addition of 1.0 ml of acetic acid (30%). The product (4-nitro aniline) thus released was determined spectrophotometrically by measuring the intensity of yellow colour 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. Amount of product (4-nitroaniline) released was calculated through a standard curve prepared in the same way with varying concentration of 4-nitroaniline and read against a suitable reagent blank.

(iv). Fluorimetric assay of 7-amino-4-methylcoumarin releasing substrates :

Enzymatic assay against 7-amino-4-methylcoumarin releasing substrates like Z-Phe-Arg-MCA, Z-Arg-Arg-MCA, Arg-MCA etc. were done according to the procedure described by Barrett and Krischke (1981), incorporating slight modification, using a spectrofluori-photometer fitted with thermostat water bath. The procedure is briefly described below:

Reagents:

(i). Activator buffer:		340 mM sodium acetate, 60 mM acetic acid 4 mM disodium EDTA, pH 6.5. 8 mM dithiothritol (DTT) was added freshly to the buffer for immediate use.
(ii) Substrate	:	10 mM of stock solution of substrates in dimethyl sulfoxide (DMSO), stored below 0°C was diluted to the required working concentrations by diluting with activator buffer without DTT.
(iii) Diluent	:	Brij 35 (0.1%) in water.
(iv) Aminomethyl coumarin standard	:	7-amino-4-methylcoumarin (1 mM) stock in DMSO, stored below 0°C. During assay the stock was freshly made to the required concentrations by diluting with the activator buffer without DTT.

Procedure :

0.5 ml of enzyme in solution in diluent was incubated with 1.0 ml of activator buffer with 8.0 mM DTT, at 37°C for 2 mins. The reaction was started by adding 0.5 ml of diluted (working) substrate solution. The increase in fluorescence was measured continuously for 30 mins by fixing excitation and emission wave-lengths at 370 nm and 440 nm respectively, using a suitable

reagent blank for calibration. The total amount of 7-amino-4-methylcoumarin liberated during enzymatic assay was calculated from the standard curve of 7-amino-4-methylcoumarin prepared in 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 minute.

b. Enzymatic assay with protein substrates :

(i). Assay with azocasein:

Enzymatic assay against azocasein was done according to the method of Barrett **et al.,** (1981).

0.25 ml of enzyme solution was incubated with equal volume of activator buffer (20mM sodium phosphate buffer, pH 6.5, containing 10 mM cysteine base and 1.0 mM each of EDTA and pepstatin) at 37°C for 10 mins. To it 0.5 ml of azocasein solution (6% stock in water, diluted to different concentrations with the activator buffer, described above but without cysteine base) was added and mixed by inversion. The reaction was allowed to occurred for 30-60 mins and stopped thereafter by addition of 5.0 ml of 10% (w/v) chilled TCA solution. The whole content was then centrifuged at 4,000 rpm at 4°C. The yellow filtrate was taken and the absorbance at 366 nm was taken against suitable blank, prepared in the same way except that the substrate solution was added to the enzyme only after addition of TCA solution. One unit of enzyme was defined as the amount of enzyme required to bring about a change in OD at 366 nm by 0.01 absorbance unit per hour.

(ii). Assay with casein, haemoglobin (Hb) and BSA :

The enzymatic assays with acid denatured protein substrates, bovine milk casein, goat Hb, buffalo Hb and BSA, were done by the method described by Moore and Stein (1954) and Ahmad **et al.,** (1990).

A highly concentrated protein solution prepared in 20 mM phosphate buffer, pH 6.5 , containing 2.0 mM each of EDTA and 2-mercaptoethanol was exposed to acetic acid at pH 2.8 and left overnight. This was then kept in a boiling water bath for about 1 hr. After cooling at room temperature, the content was centrifuged and filtered through a layer of whatman #1 filters. The filtrate was taken and excessively dialysed against the phosphate buffer described above.The substrate concentration was finally adjusted to the desire range by diluting it with the same buffer at pH 6.5.

To 0.5 ml of enzyme solution in activator buffer, preactivated at 37°C for 30 mins was added 0.5 ml of the substrate solution (prepared as above) and mixed properly. After 3 hrs of incubation at 37°C, the reaction was terminated by adding 1.0 ml of chilled TCA (30% w/v) and the content was centrifuged for 10 mins at 4,000 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 (prepared freshly by dissolving 0.3 gms of hydrindantin and 2.0 gms of ninhydrin in 4.0 M sodium acetate buffer, pH 5.5, to a volume of 25.0 ml followed by

dilution to a final volume of 100.0 ml with addition of methyl-cellusolve) and incubated at 80°C for 20 mins. After cooling the solution under tap water, 5.0 ml of ethanol was slowly added and the intensity of the blue colour thus formed was measured at 570 nm using the suitable blank prepared in the same way except that the protein substrate was added after addition of 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 OD per hour under our assay conditions.

c. Determination of catalytic parameters :

The values of K_m and V_{max} of cathepsin B for both synthetic as well as protein substrates were computed using the least square 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 with the assumption that the enzymatic reaction provided the accurate values for the K_m for the substrate concentration between 20-80 % saturation.

11. Determination of specific extinction coefficient :

Concentrated protein solution was first repeatedly dialysed against excess of distilled water followed by passing through a column of mixed-bed resin of Amberlite MB-3. The pH of the effluent containing the protein solution was directly measured to give its isoionic pH. Absorbance of this solution was measured at 280

nm. Known volumes of this solution were taken in a set of pre-weighed weighing bottles. The contents in these bottles were heated to dryness at 110°C. The bottles were weighed repeatedly (after alternate heating and cooling) at fixed interval of time until the constant weight was obtained. The exact weight of the protein taken in each bottle was determined by subtracting the weights of the empty bottles from that of the respective bottles containing the dry protein. The specific extinction coefficient ($E^{1\%}_{1cm}$) of the protein was thus calculated by dividing the optical density of the protein solution by its weight (gm/100ml).

12. Measurement of intrinsic viscosity :

Measurement of viscosity of the purified cathepsin B was done in a Schott Gerate (Type 513 00) viscometer having a flow time of about 430 sec for 3.0 ml distilled water at 25°C.

Clean and air dried viscometer was placed in an insulated glass water-bath fitted with a thermostat (HAAKE, model D8) maintained at 25°C. 3.0 ml of protein solution (previously dialysed in appropriate buffer and passed through millipore filter pore size (0.45 μM) of varying concentration (0-2 mg/ml) were placed 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

computed by the method of Tanford (1955) using the following expression:

$$[\eta] = \lim_{C \to 0} (\eta - \eta_0)/\eta_0\ C$$
$$= \lim_{C \to 0} [(t - t_0) / t_0\ C] + (1 - \bar{V}_2 \rho_0 / \rho_0)$$

where, η_0 and η were the viscosities in poise of the solvent (buffer) and the protein solution respectively, C was the protein concentration in gm/ml, ρ_0 was the density of the solvent, and $\bar{V}_2$ was the partial specific volume of the protein.

13. Immunological Studies :

Polyclonal antibodies were raised against purified cathepsin B in rabbits (Himalayan albino) by using standard immunization protocols. About 100 µg of the purified (and extensively dialysed against distilled water) protein conjugated with complete Freund's adjuvent was injected intramuscularly followed by two booster doses (in incomplete Freund's adjuvent) given at an interval of 30 and 60 days. Sera was collected after 7 days of the second booster. The cross reactivity of the antisera against cathepsin B isolated from different batches and sources was checked by Ouchterlony double diffusion technique (Ouchterlony, 1949). Immunodiffusion was performed both at pH 6.8 and pH 8, using 2% (w/v) agarose gel at 37°C.

Immunoinhibition of the purified enzyme was also performed using the antisera following the substrate depletion assay method (Coetzer **et al.,** 1991; Rowan **et al.,** 1992) using BANA as substrate. 0.4 ml of cathepsin B (0.4 µg) or cathepsin H (0.5 µg)

were incubated at 37°C for 1 hr. with 0.1 ml of antiserum produced against buffalo kidney cathepsin B or normal rabbit serum (NRS). Dilution of antiserum was done with normal rabbit serum. The residual BANA-lyase activity was measured colorimetrically following the procedure described previously.

RESULTS

A. Isolation and purification of cathepsin B :

Cathepsin B was isolated from buffalo kidney according to the method of Ahmad **et al.,**(1989) and purified incorporating suitable modifications in the procedure described by Takahashi **et al.,** (1984a). The protein fraction obtained at 40-75% ammonium sulfate saturation was subjected to gel chromatography on a Sephadex G-75 column (2.6x95 cm) equilibrated with 50 mM sodium acetate buffer, pH 5.0, containing 1 mM EDTA and 0.02% sodium azide and the elution profile thus obtained is given in Fig.4. The fraction exhibiting appreciable BANA hydrolase activity (indicated by the horizontal bar) were pooled and subjected to ion exchange chromatography on a CM-Sephadex cation exchanger column (1.6x12 cm), pre-equilibrated with 20 mM sodium acetate buffer, pH 4.8 containing 1mM EDTA and 1.4 mM 2-mercaptoethanol. The bound protein fraction was eluted from the column by stepwise application of the same buffer at pHs 4.8, 5.6, 6.0 and finally using the sodium chloride gradient (0-1.0 M) at pH 6.0 (Fig. 5). Fractions eluted with 20 mM sodium acetate buffer, pH 5.6 showed appreciable BANA and Z-Arg-Arg-MCA-lyase activities, characteristic of cathepsin B, while cathepsin H like activity was confined only to the fractions eluted between 0-0.5 M NaCl gradient in the same buffer at pH 6.0. Protein peak obtained at pH 6.0 did not show any activity against BANA, Z-Arg-Arg-MCA and Leu-NA or Arg -NA.

Fig. 4. Elution profile of the $(NH_4)_2SO_4$ fraction (40-75% saturation of buffalo kidney cathepsin B in Sephadex G-75 column.
About 150 mg of protein was applied in the column (2.6x90 cm) equilibrated with 50 mM sodium acetate buffer, pH 5.0 containing 1 mm EDTA and 0.02% sodium azide. Protein was eluted in 5.0 ml fractions was monitored at 280 nm for protein (o---o) and at 540 nm for BANA lyase activity (o—o). Horizontal bar indicates the active fractions pooled for further purification.

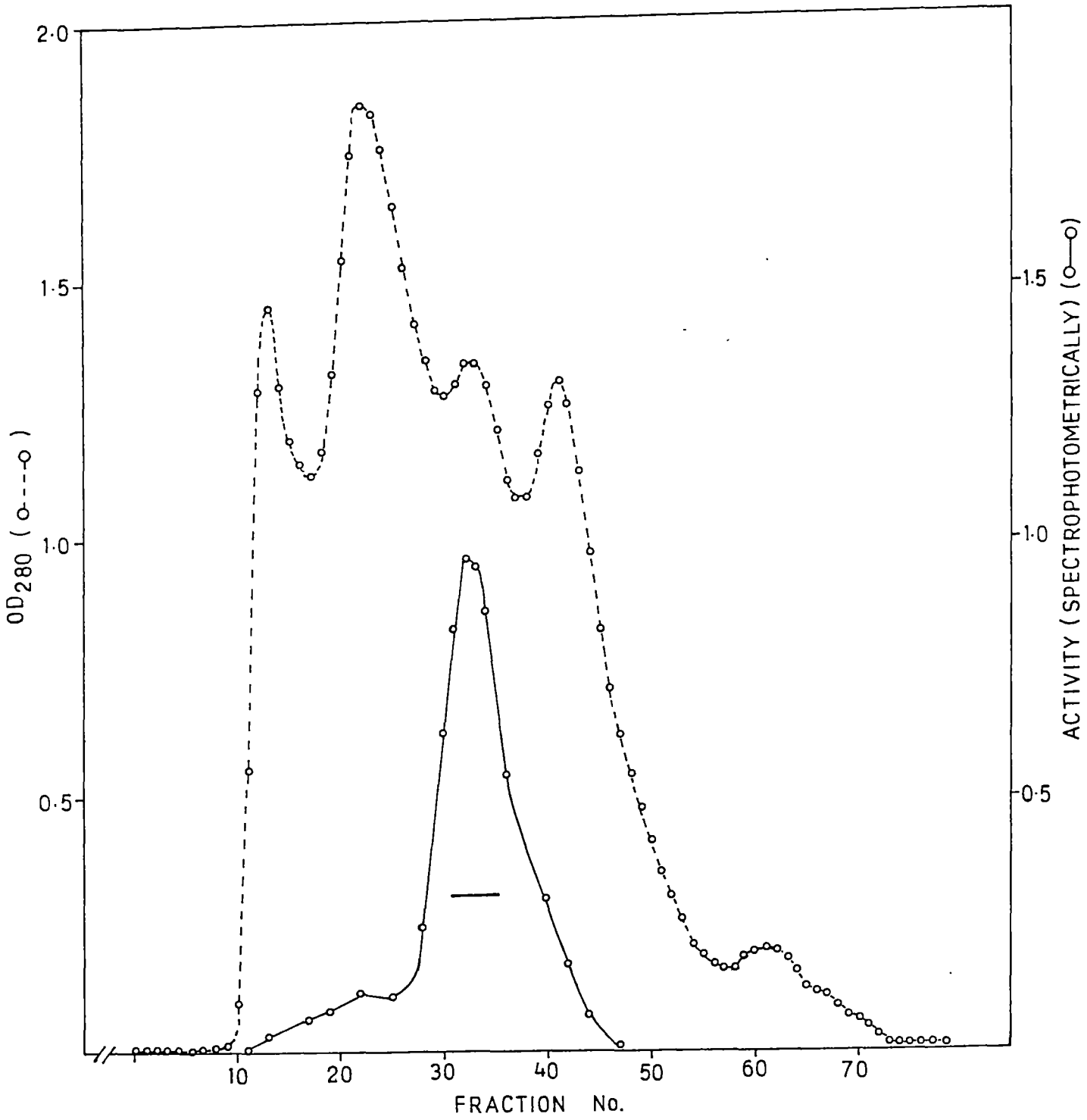
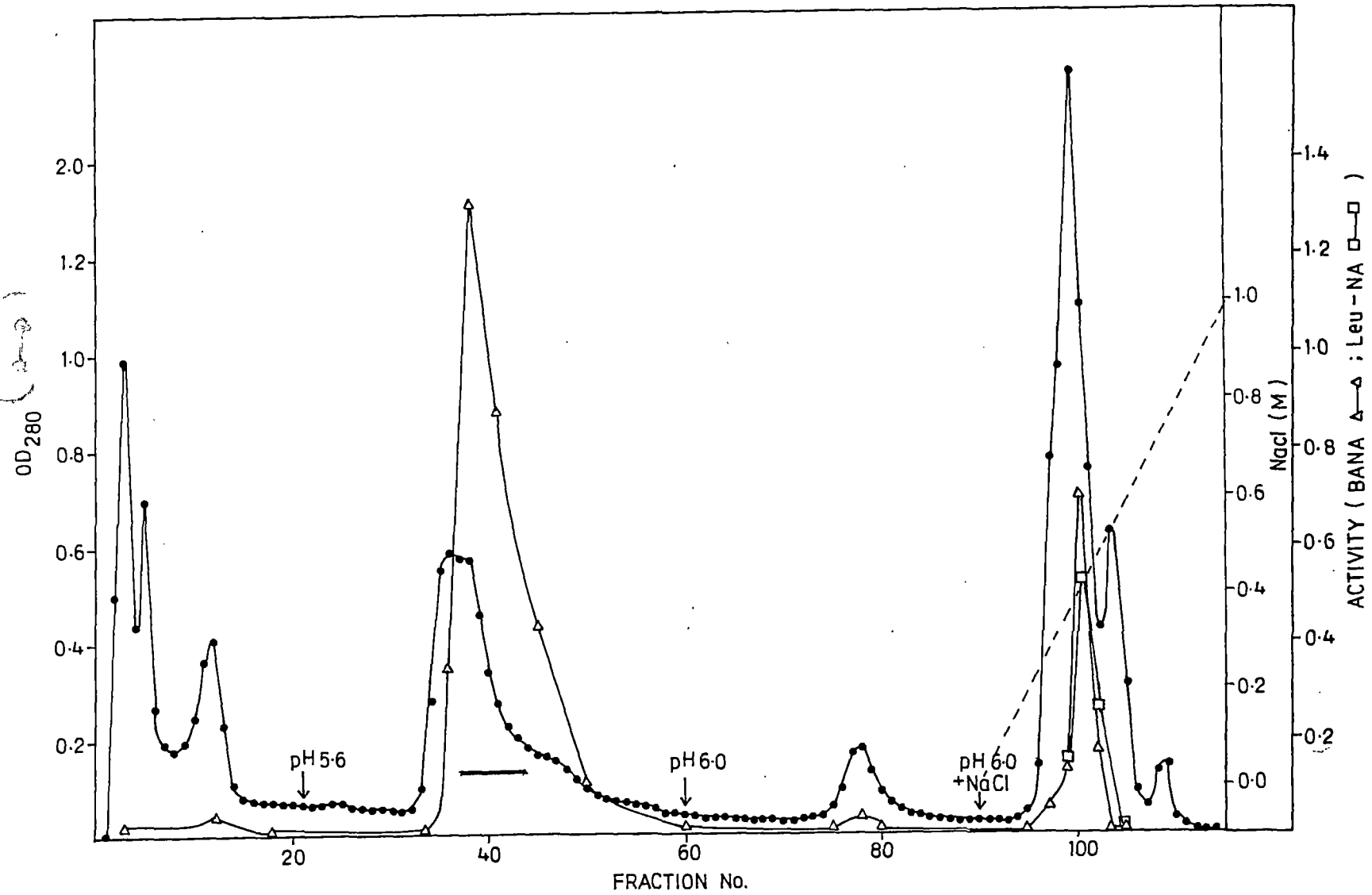

2·0
1·5
1·0
0·5
OD280 (o---o)
1·5
1·0
0·5
ACTIVITY (SPECTROPHOTOMETRICALLY) (o—o)
10
20
30
40
50
60
70
FRACTION No.

Fig. 5. Elution profile of the active fraction of the cathepsin B (obtained through the gel filtration) in CM-Sephadex C-50 cation exchange column.
About 50 mg of protein was applied in 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 developed with the same buffer at pH 4.8 followed by step-wise elution at pH 5.6, 6.0 and finally using sodium chloride gradient (0-1.0 M). Protein concentration was measured at 280 nm (●——●). Activity was determined using BANA (△——△) and Leu-NA (□——□). Horizontal bar shows the cathepsin B active fractions pooled for the purification.


OD_{280}
2·0
1·2
1·0
0·8
0·6
0·4
0·2
pH 5·6
pH 6·0
pH 6·0 +NaCl
20
40
60
80
100
FRACTION No.
1·4
1·2
1·0
0·8
0·6
0·4
0·2
0·0
Nacl (M)
ACTIVITY (BANA Δ—Δ ; Leu-NA □—□)

The pH 5.6 fractions with appreciable cathepsin B like activities were pooled, concentrated and dialysed excessively against 25 mM histidine buffer pH 6.25, containing 1 mM EDTA and 1.4 mM 2-mercaptoethanol. Lyophilization of sample at this stage was omitted as it resulted in significant loss of activity. The concentrated and dialysed protein solution was subjected to chromatofocusing on a PBE 94 column (0.9x12 cm) pre-equilibrated with the above mentioned histidine buffer and eluted with decreasing pH gradient by applying polybuffer 74 (diluted 1:8) adjusted to pH 4.0. Cathepsin B eluting out between pH 5.2 and 4.8 (Fig. 6) was salted out with ammonium sulfate and subjected to rechromatography on a Sephadex G-100 column chromatography. The latter step was essential for the removal of polybuffer, autolysed products and other probable contaminants.

Test of homogeneity :

The enzyme preparation thus obtained was found to be homogeneous with respect to size and charge as evident from the analytical gel chromatography and non-denaturing polyacrylamide gel electrophoresis (PAGE) studies. The protein peak obtained through analytical gel filtration appeared to be single symmetrical peak fully superimposable with its activity peak (Fig. 7). The PAGE pattern of the enzyme on 7.5% gel at pH 8.3 resulted in a single prominent protein band (Fig. 8). This is in contradiction to the reports by earlier workers who questioned the homogeneity of the enzyme (cathepsin B) based on charges (Takahashi

Fig. 6. Chromatofocusing of cathepsin B (fraction obtained from CM-Sephadex C-50 Chromatography) in PBE-94 column (0.9x12 cm).

About 20 mg of protein was subjected to the column pre-equilibrated with 25 mM histidine buffer, pH 6.3, containing 1 mM EDTA and 1.4 mM 2-mercaptoethanol. The protein was eluted with PB-74, pH 4.0. Cathepsin B eluted between pH 5.2-4.8.

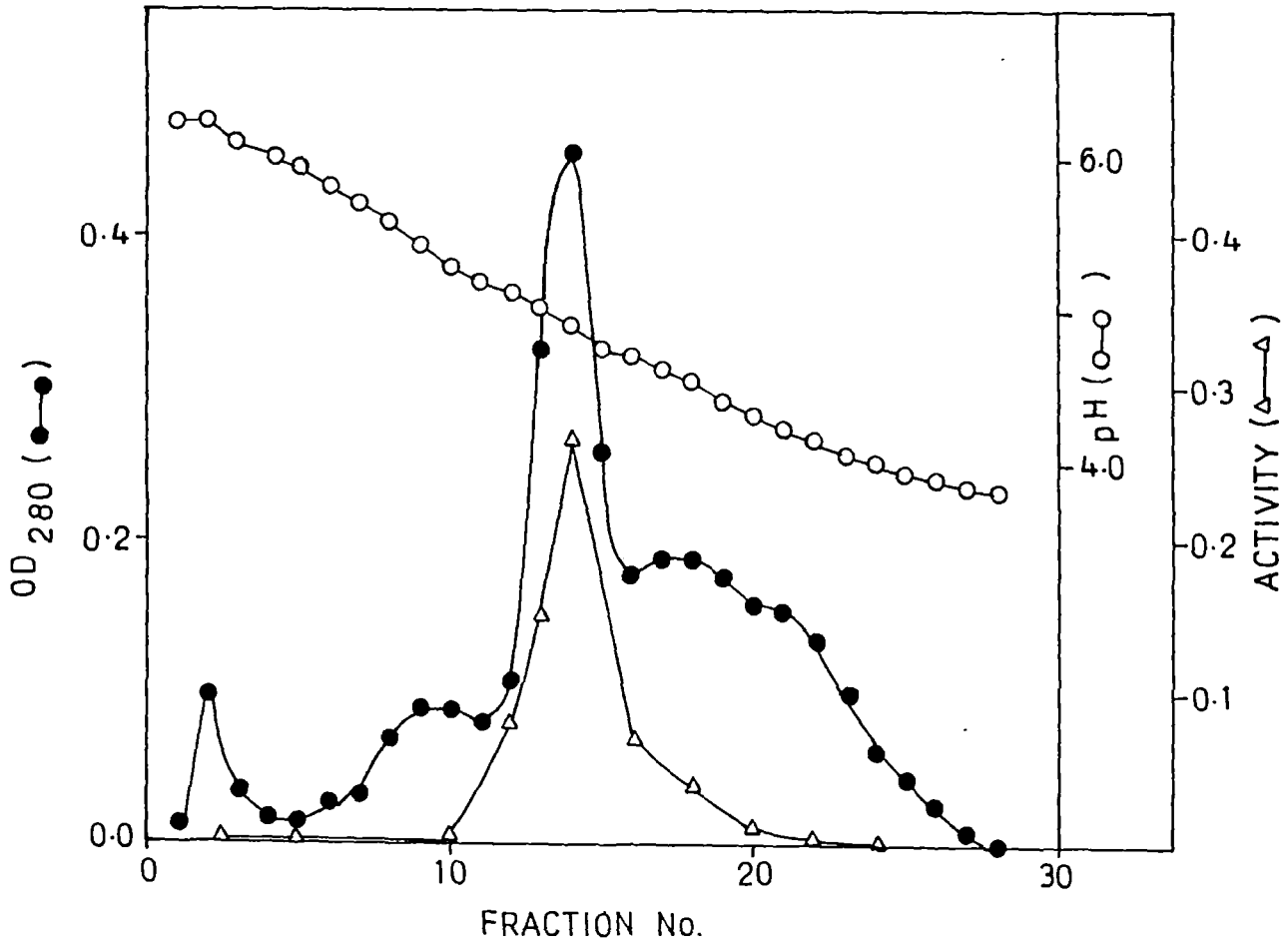

OD 280 (●—●)
0·4
0·2
0·0
0
10
20
30
FRACTION No.
6·0
4·0
pH (○—○)
0·4
0·3
0·2
0·1
ACTIVITY (△—△)

Fig. 7. Rechromtographic profile of cathepsin B (after chromatofocusing in PBE-94) in Sephadex G-100 column (1.6x 92 cm).
About 20 mg of protein was supplied to the column equilibrated with 60 mM phosphate buffer, pH 6.0, containing 1 mM EDTA and 0.02% sodium azide. Protein was eluted at a flow of 15 ml/hr. Protein was monitored at 280 nm (⊙——⊙) and activity was assayed using Z-Arg-Arg-MCA (□——□).

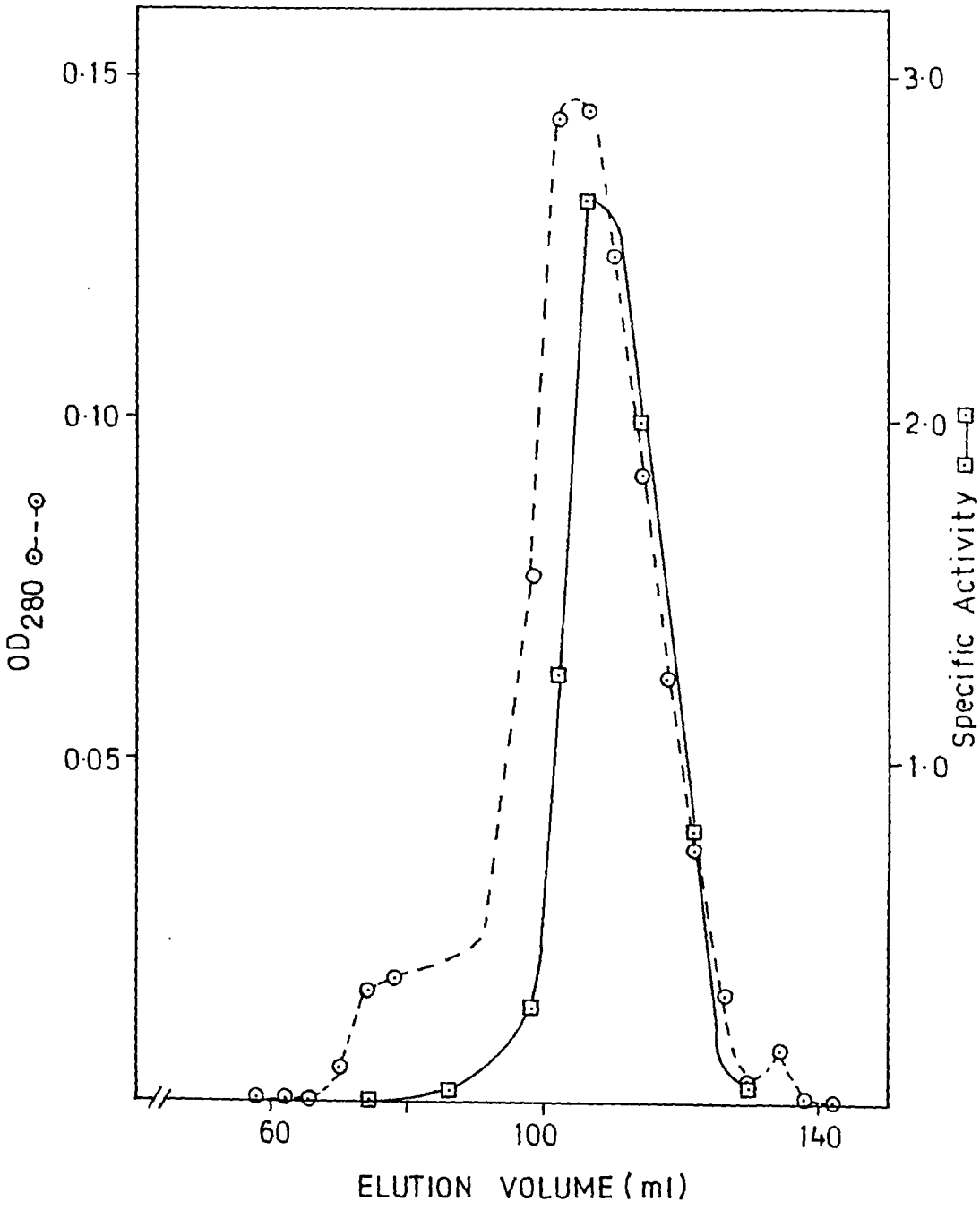

0·15
0·10
0·05
OD_{280}
3·0
2·0
1·0
Specific Activity
60
100
140
ELUTION VOLUME (ml)

TABLE II : Purificaton of cathepsin B from buffalo kidney *

Purification Steps	Total protein (mg)	Total Enzyme Activity (IU)	Specific Activity (IU/mg)	Purification Fold	% Yield
Crude Extract	22846.00	379.14	0.016595	1.00	100.00
Acid Extraction	1937.58	153.65	0.07929	4.78	40.52
Ammonium Sulfate Fractionation	502.33	93.76	0.186648	11.25	24.73
Sephadex G-75 Chromatography	144.20	73.65	0.510776	30.79	19.43
CM-Sephadex Chromatography	32.60	55.39	1.69896	102.34	14.62
Chromatofocusing followed by Re-chromatography on Sephadex G-100	3.57	9.55	2.78700	167.97	02.62

*Data obtained from 600 gm of buffalo kidney.

Fig. 8. Polyacrylamide gel electrophoretic pattern of the purified buffalo kidney cathepsin B using 0.02 M Tris-glycine buffer, pH 8.3.
About 60 µg of cathepsin B was applied on the gel (7.5%) and electrophoresis was done for about 2 hrs. with a constant current of 3 mA/tube. The gels were stained in amido black and destained with a mixture of 7% acetic acid in 5% methanol.


−
Dye
+

et al., 1986; Fazilli and Qasim,1986; Agarwal and Khan, 1987(b); Ahmad **et al.,**1989).

The methodology developed and applied here was found to be simple and could be used for simultaneous isolation and purification of cathepsin B and H from the same source at a significantly higher activities and yield (Tab. II)

B. Characterisation of cathepsin B :

1. Physical characterisation :

a. Molecular Weight determination :

(i). Gel filtration :

The molecular weight of cathepsin B was determined by gel chromatography on a Sephadex G-100 column (1.6x92 cm) calibrated with a few standard marker proteins. Figure 9 and 10 show the elution profile of the various marker proteins used for calibration of the column as well as that of the purified cathepsin B. The void volume (V_0) of the column as determined by passing Blue Dextran 2000 through the column came out to be 74.0 ml. The distribution coefficient (K_{av}) were calculated using the following equation :

$$K_d = (V_e - V_0) / V_i$$

$$K_{av} = (V_e - V_0 / (V_t - V_0)$$

where V_t and V_i are the total and the inner volumes of the column, respectively. The inner volume (V_i) of the gel was calculated from the elution volume of $K_3Fe(CN)_6$ using the equation

$$V_i = V_p - V_0$$

Fig. 9. Chromatography of various marker proteins on the calibrated Sephadex G-100 column (1.6x92 cm). About 5-10 mg of each sample was applied to the column equilibrated with 60 mM phosphate buffer pH 6.0. Protein samples applied were : (1) BSA, (2) Ovalbumin, (3) Carbonic anhydrase, (4) Chymotrypsinogen A, (5) Cytochrome C.

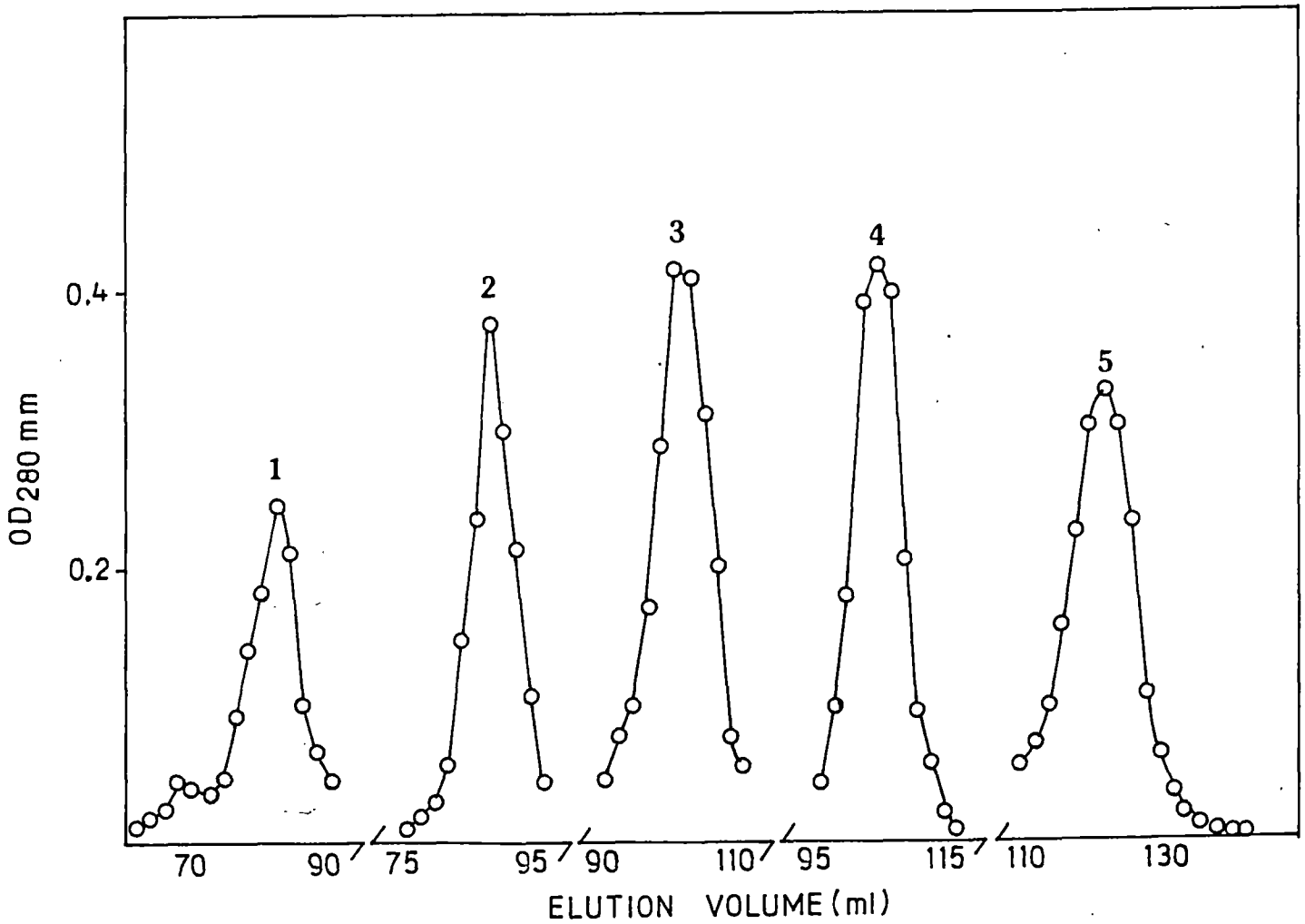

0.4
0.2
OD280 mm
1
2
3
4
5
70
90
75
95
90
110
95
115
110
130
ELUTION VOLUME (ml)

Fig. 10. Chromatography of Blue Dextran 2000, buffalo kidney cathepsin B and K_3 $Fe(CN)_6$ in the calibrated Sephadex G-100 column (1.6x92 cm).

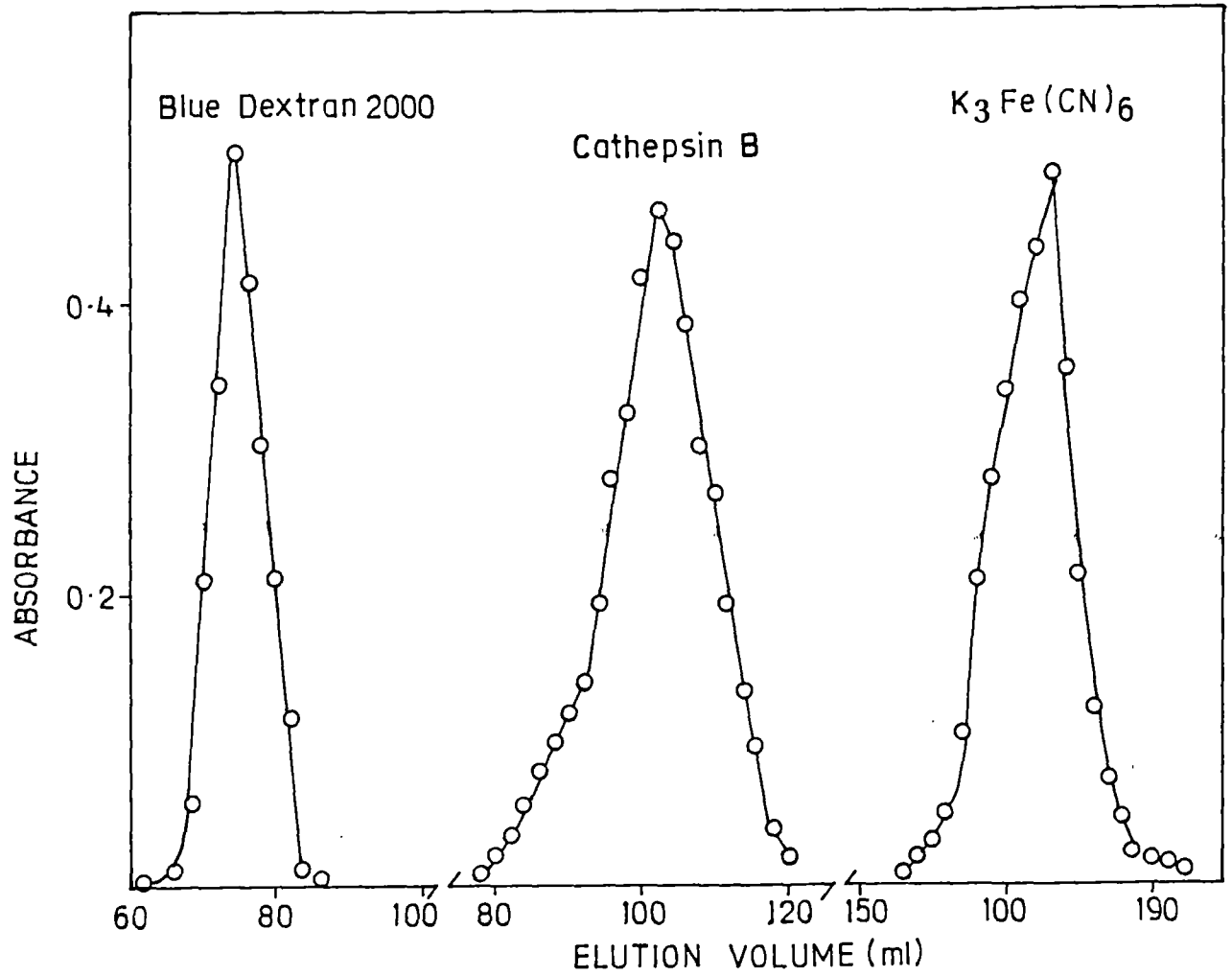

Blue Dextran 2000
Cathepsin B
$K_3 Fe(CN)_6$
0·4
0·2
ABSORBANCE
60
80
100
80
100
120
150
100
190
ELUTION VOLUME (ml)

TABLE III : Some hydrodynamic parameters of standard marker proteins and buffalo kidney cathepsin B from analytical gel filtration

Protein	V_e/V_o	K_d	$K_d^{1/3}$	K_{av}	$(-\log K_{av})^{1/2}$	$erfc^{-1}K_d$
Bovine serum albumin (BSA)	1.1081	0.07843	0.4280	0.0721	1.0688	1.2445
Ovalalbumin	1.1891	0.13725	0.5158	0.1261	0.9483	1.0479
Carbonic anhydrase	1.3648	0.26470	0.6421	0.2432	0.7836	0.7891
Chymotrypsin-ogen A	1.4054	0.29412	0.6650	0.2703	0.7538	0.7425
Cytochrome C	1.6486	0.47059	0.7778	0.4328	0.6034	0.5105
Cathepsin B	1.3784	0.2745	0.6409	0.2523	0.7734	0.7722

where V_p is the elution volume of $K_3Fe(CN)_6$, which came out to be 176 ml. V_i was thus found to be 102 ml. The total volume V_t for the column (1.6x92 cm) as deduced from the formula $\pi r^2 l$ was 185 ml. The values of K_d and K_{av} for the buffalo kidney cathepsin B were obtained by treating the data according to Andrews (1970) and Porath (1963) and were found to be 0.2745 and 0.25225 respectively (Tab. III). The average molecular weight for this enzyme was thus found to be about 27 kDa (Fig.11 & 12 and Tab. IV).

(ii). Sodium dodecyl sulfate polyacrylamide gel electrophoresis :

Determination of molecular weight of purified buffalo kidney cathepsin B was also performed using sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) on 10% polyacrylamide gels containing 1.5% SDS, according to the method of Laemmli (1970), (Fig.13 & 14). Table V lists the relative mobilities (R_m) of the different marker proteins as well as the purified cathepsin B and their corresponding log of molecular weight. A plot of the R_m and the corresponding log of molecular weight of the marker proteins resulted in a straight line yielding an apparent molecular weight of about 25.5 kDa for the purified cathepsin B (Fig. 15).

Hence, putting together the values obtained by analytical gel filtration and SDS-PAGE, buffalo kidney cathepsin B has been assigned a molecular weight of about 26 kDa, which falls well within range (23-29 kDa) as reported earlier from other sources

Fig. 11 Plot of V_e/V_0 versus log M.
The number of marker proteins were in accordance with legend to figure 9. The straight line is drawn by the method of least square that fits the equation,
$\log M = 6.2442 - 1.309\ V_e/V_0$.

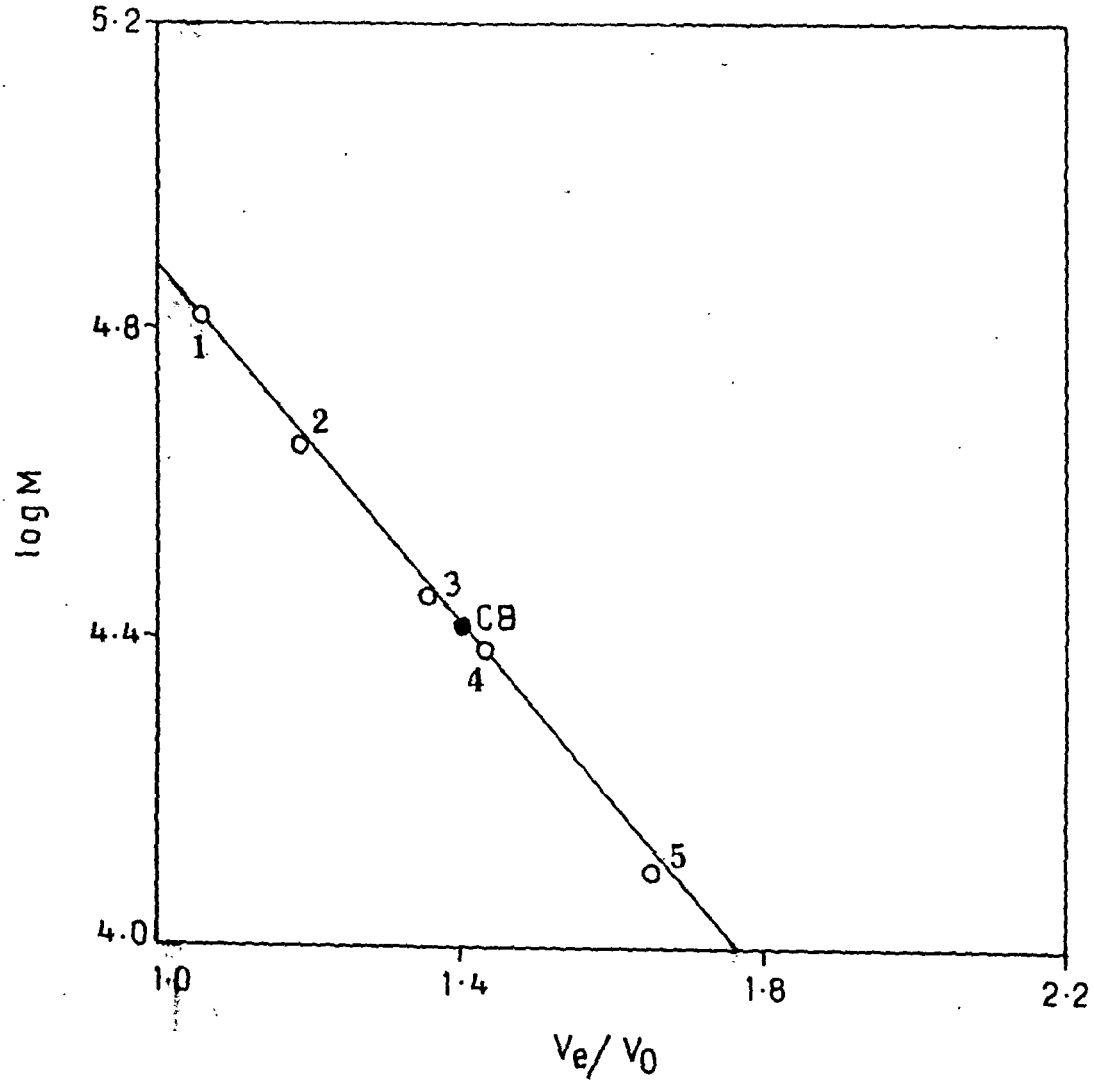

5·2
4·8
4·4
4·0
log M
1
2
3
CB
4
5
1·0
1·4
1·8
2·2
Ve/ V0

Fig. 12. Determination of molecular weight according to method of Porath (1963). The marker proteins were same as described in legend to figure 9. The best fit plot fits the equation,
$MW^{1/3} = 60.886 - 47.95\ K_d^{1/3}$.

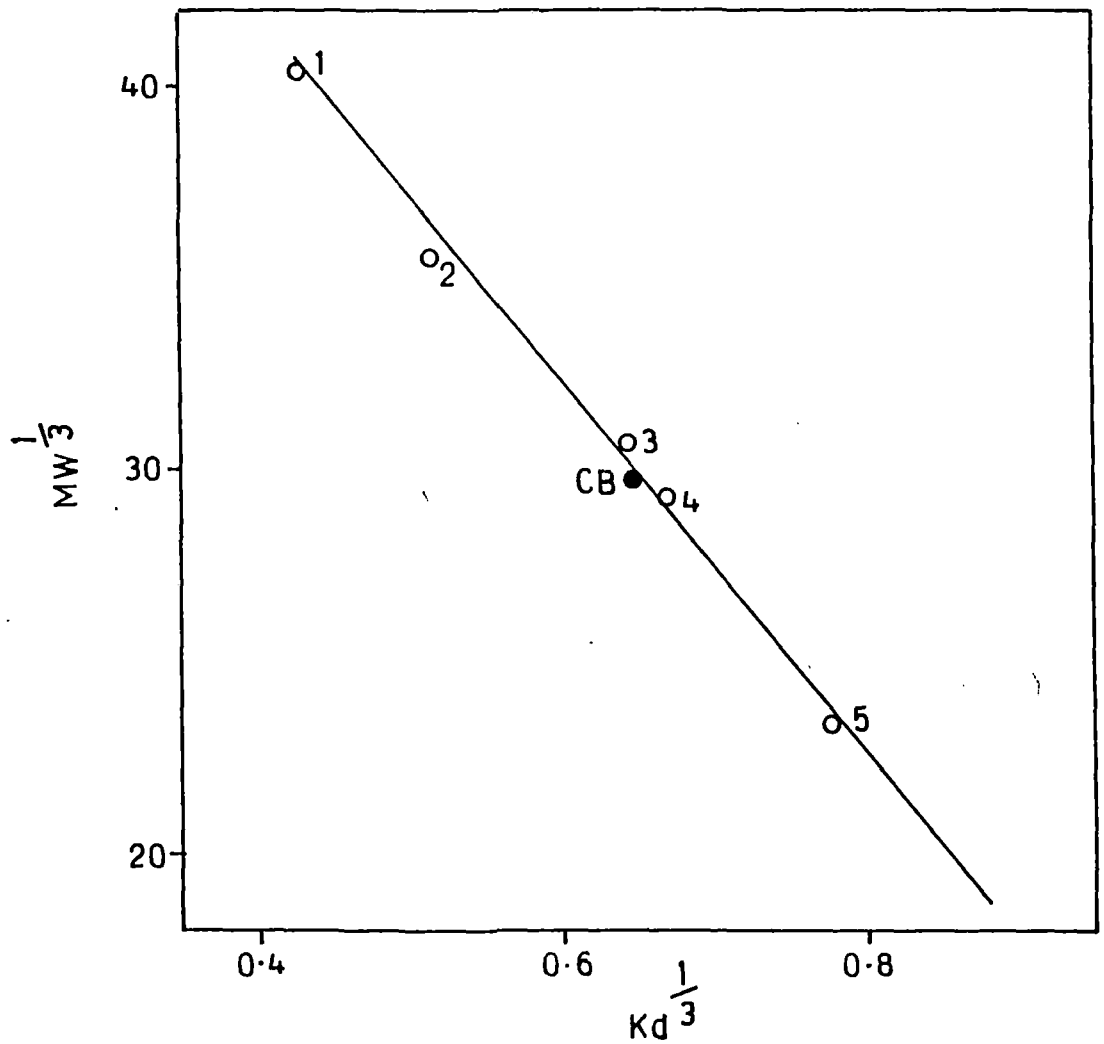

40
30
20
$MW^{\frac{1}{3}}$
1
2
3
CB
4
5
0·4
0·6
0·8
$Kd^{\frac{1}{3}}$

TABLE IV : Molecular weight, stokes radii and geometric mean radii of marker proteins and buffalo kidney cathepsin B in 60 mM phosphate buffer, pH 6.0

proteins	Molecular Weight[a] (kDa)	Stokes radius[b] (nm)	Geometric mean[c] radius (nm)
Bovine serum albumin	66.5	3.50	2.67
Ovalbumin	45.0	3.00	2.31
Carbonic anhydrase	29.0	2.37	-
Chymotrypsinogen-A	25.0	2.20	1.95
Cytochrome C	12.4	1.70	1.51
Cathepsin B[d]	27.0	2.32	2.05

a : Tanford **et al.**, 1968
b : Tanford **et al.**, 1974
c : Suelter **et al.**, 1985
d : Determined in the present study

Fig. 13. Electrophoretic pattern of buffalo kidney cathepsin B in SDS-polyacrylamide gel (10%).
About 50 µg of protein was applied in the gel containing 1.5% SDS and 0.1% 2-mercaptoethanol. Electrophoresis was carried for 2.5 hrs. using 60 mM Tris-HCl buffer, pH 8.3, containing 1.5% SDS. A constant current of 2.5 mA/tube was applied.


Dye

Fig. 14. Electrophoretic pattern of marker proteins and buffalo kidney cathepsin B in SDS polyacrylamide gels (10%). The marker proteins were : (1) BSA, (2) Ovalbumin, (3) Chymotrypsinogen-A, (4) Myoglobin and (5) Lysozyme. Cathepsin B is marked as CB. Other experimental details were same as described in legend to figure 13.

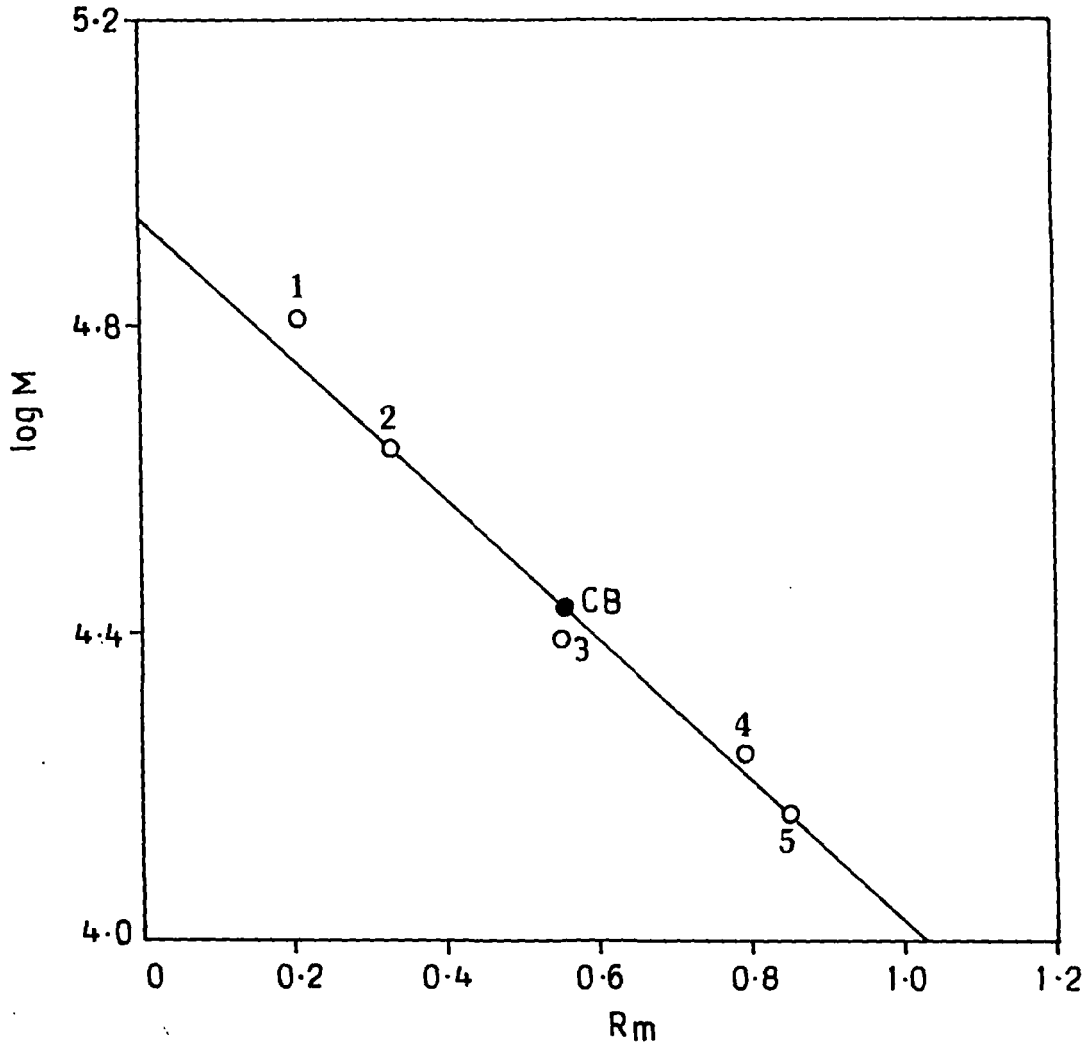

5 4 3 2 1 CB
Dye

TABLE V : Molecular weight and R_m values of marker proteins and buffalo kidney cathepsin B on SDS-PAGE (10%)

Sl. No.	Proteins[a]	Mol. Wt. (kDa)	log Mol. Wt.	R_m
1.	Bovine Serum Albumin (BSA)	66.5	4.8228	0.2127
2.	Ovalbunin	45.01	4.6532	0.3296
3.	Chymotripsino-gen-A	25.00	4.3979	0.5532
4.	Myoglobin	17.80	4.2504	0.7979
5.	Lysozyme	14.80	4.1702	0.8511
6.	Cathepsin B	25.50[d]	-	0.5430

a: Values taken from Weber and Osborn (1969).
d: Determined in this study.

Fig. 15 Determination of molecular weight of buffalo cathepsin B by SDS-polyacrylamide gel electrophoresis. The labelings of marker proteins and cathepsin B are in accordance with legend to fig. 14. The straight line was plotted according to least squares method which follows the equation,
$\log M = 4.9328 - 0.968R_m$


5·2
4·8
4·4
4·0
log M
1
2
CB
3
4
5
0
0·2
0·4
0·6
0·8
1·0
1·2
Rm

(Bajkowski and Frankfater, 1982; Barrett and Kirschke, 1981; Takahashi **et al.**, 1984; Hirao **et al.**, 1984; McDonald and Barrett, 1986; Fazili and Qasim, 1986; Agarwal and Khan, 1987; Ahmad **et al.**, 1989). However, our result do not agree well with the two chain form of the enzyme as only one prominent band was obtained in SDS-PAGE studies both in presence and absence of 2-mercapto-ethanol (Fig. 13).

b. Hydrodynamic properties of cathepsin B :

(i) Stokes radius:

Stokes radius of the buffalo kidney cathepsin B was determined from the annalysis of the data obtained from the analytical gel filtration in Sephadex G-100 column (1.6x92 cm) according to Laurent and Killander (1964) and Ackers (1967). The results have been summarised in figure 16 & 17 yielding a value of 2.32 and 2.326 nm respectively. The avarage value of the stokes radius for the buffalo kidney thus found to be 2.32 (Tab. IV).

Computation of geometric mean radius by the method of Ackers as described by Suelter(1985) yielded a value of 2.057 (Fig. 18).

(ii) Viscosity :

The intrinsic viscosity of the purified buffalo kidney cathepsin B was determined at 25°C in 60 mM phosphate buffer, pH 6.0. The value of the intrinsic viscosity $[\eta]$ as calculated from

Fig. 16. Determination of stokes radius according to method of Laurent and Killander (1964)
The marker proteins from 1-5 were the same as described in the legend to fig 9. The filled circle (CB) indicates the position of cathepsin B. The straight line drawn by the method of least squares yields stokes radius value of 2.326 nm for buffalo kidney cathepsin B.

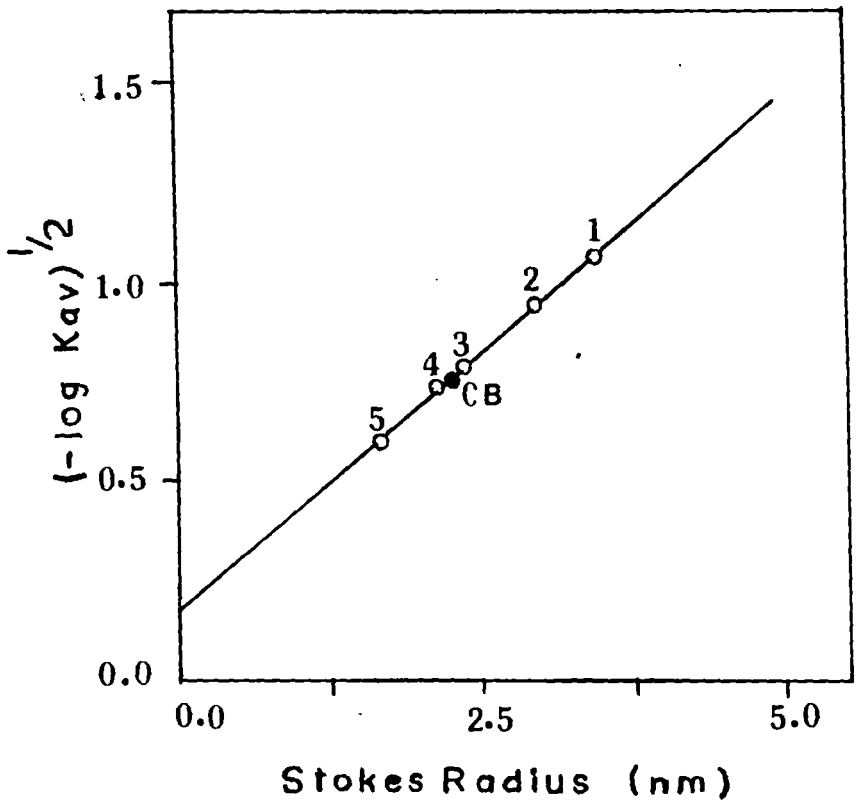

1.5
1.0
0.5
0.0
(-log Kav)^1/2
0.0
2.5
5.0
Stokes Radius (nm)
1
2
3
4
5
CB

Fig. 17. Determination of stokes radius according to method of Ackers (1967).
The marker proteins from 1-5 were the same as described in the legend to fig. 9. The filled circle (CB) indicate the position of cathepsin B. The straight line was drawn by using least squares method, which follows the equation,
$erfc^{-1}K_d = 0.40546r - 0.1707$.

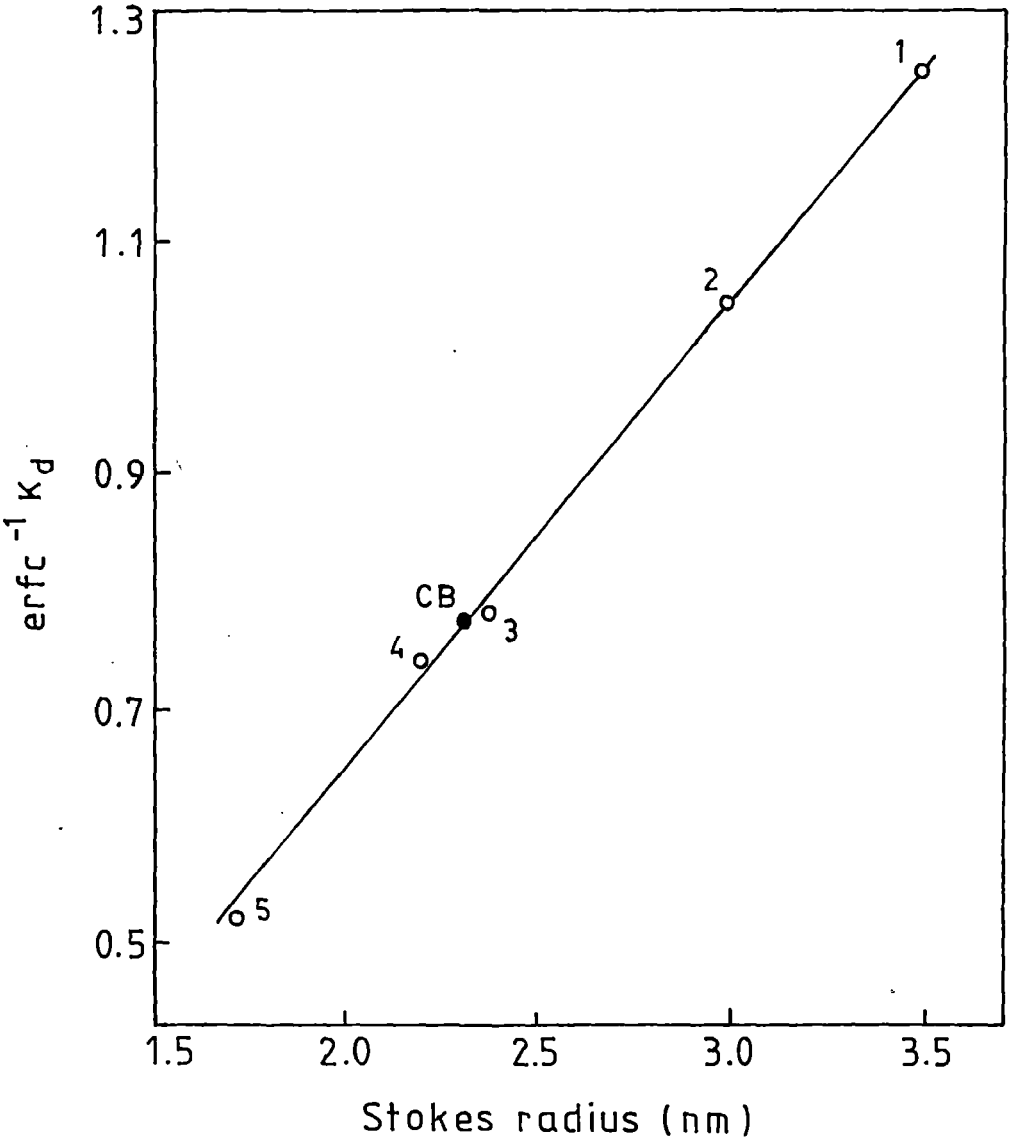
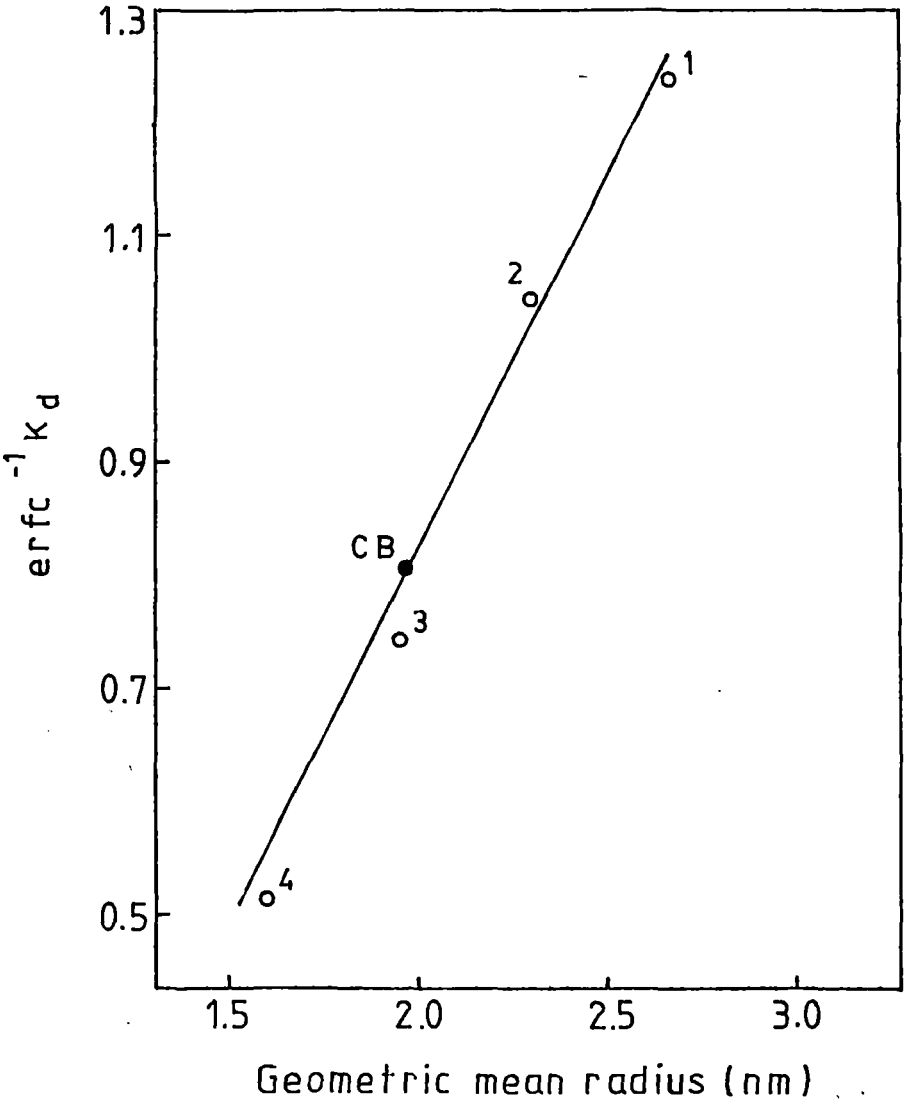

1.3
1.1
0.9
0.7
0.5
erfc^{-1} K_d
1.5
2.0
2.5
3.0
3.5
Stokes radius (nm)
1
2
CB
3
4
5

Fig. 18. Determination of geometric mean radius of buffalo kidney cathepsin B.
The marker proteins were in accordance with the legend to fig. 9. Filled circle indicate the position of cathepsin B. The straight line was drawn by using the least squares method, that follows the equation,
$\text{erfc}^{-1} K_d = 0.65122\ r' - 0.4875$


1.3
1.1
0.9
0.7
0.5
erfc^{-1} K$_d$
1
2
CB
3
4
1.5
2.0
2.5
3.0
Geometric mean radius (nm)

the eqution described in the method section, was found to be 3.4 ml/gm (Fig. 19).

c. Optical properties :

Absorption spectra and the fluorescence emission spectra (Fig. 20) of purified buffalo kidney cathepsin B was measured in 60 mM phosphate buffer, pH 6.0 containing 1 mM EDTA salt and 0.02% sodium azide. The absorption maxima was observed at about 279 nm. The emission maxima was obtained at 335 nm, typical of protein containing high amount of tryptophan and tyrosine residues (Wetlaufer, 1962; Mach **et al.**, 1992; Horowitz **et al.**, 1992). The extinction coefficient ($E^{1\%}_{1cm}$) of the kidney enzyme was found to be 16.78. This value was higher than its counterpart from buffalo spleen (13.2), but, lower than that from human liver.

2. Chemical characterization :

a. End group analyses :

Identification of NH_2 -terminal amino acid residue of kidney cathepsin B was done according to dansylation method of Gray (1967) using thin layer chromatography on polyamide sheets. Table VI shows the relative front (R_f) values of standard amino acids in various solvent systems. The R_f values for the dansyl-derivative of the NH_2-terminal amino acid residue of the purified cathepsin B are summarised in the table VII, which corresponds to that of alanine under similar conditions and solvent systems. Thus, the NH_2-terminal amino acid of the purified buffalo kidney cathepsin B was alanine. This result does not agree with earlier results on cathepsin B from other sources (Takio **et al.**, 1983;

Fig. 19. Reduced viscosity of the purified buffalo kidney cathepsin B as a function of protein concentration at 25°C in 60 mM sodium phosphate buffer, pH 6.0. Computation of reduced viscosity $[\eta]$ by the least squares method was found to be 3.4 ml per gm.

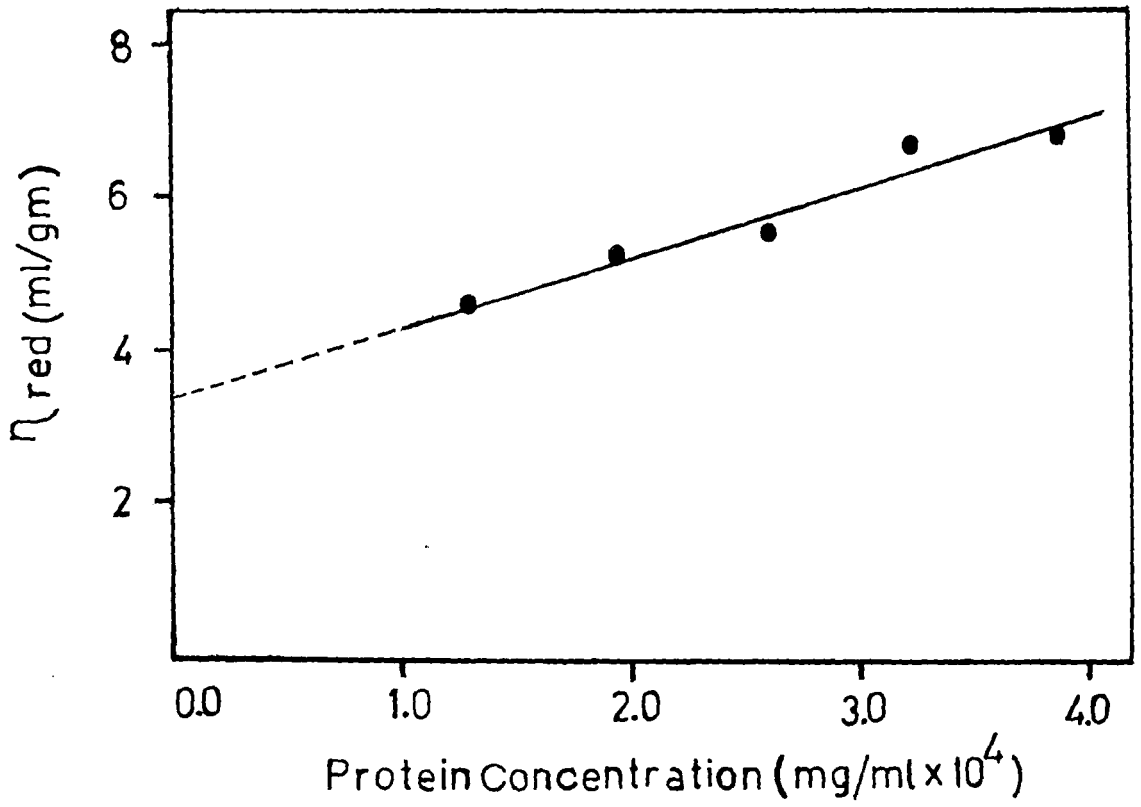

8
6
4
2
η_{red} (ml/gm)
0.0
1.0
2.0
3.0
4.0
Protein Concentration (mg/ml x 10^4)

Fig. 20 UV absorption spectrum and fluorescence emission spectrum of buffalo kidney cathepsin B in 60 mM phosphate buffer pH 6.0.
(A) UV absorption spectrum, that shows the absorption maxima at 279 nm.
(B) Fluorescence emission spectrum, which shows the emission maxima at 335 nm.

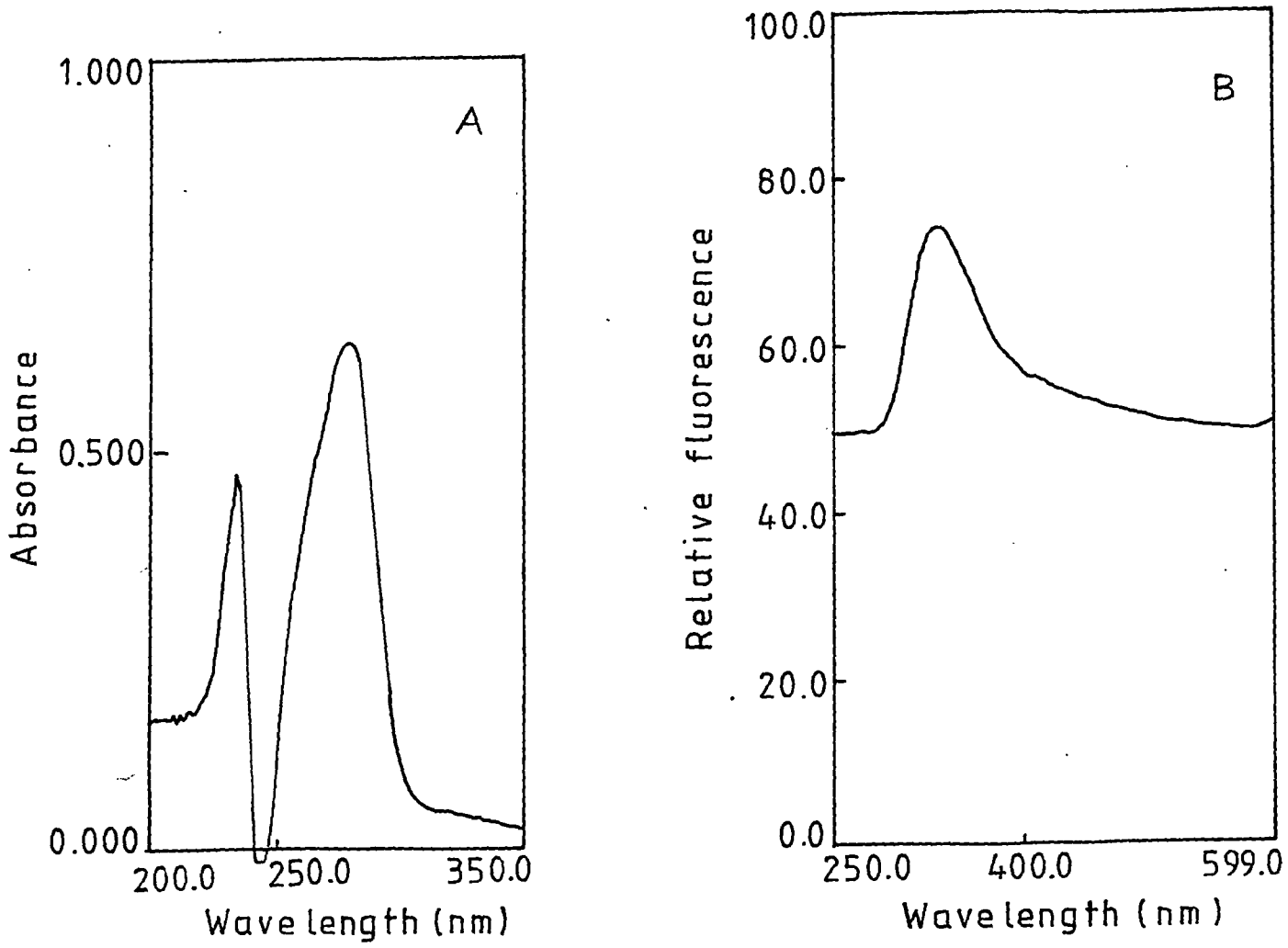

A
1.000
0.500
0.000
Absorbance
200.0
250.0
350.0
Wavelength (nm)
B
100.0
80.0
60.0
40.0
20.0
0.0
Relative fluorescence
250.0
400.0
599.0
Wavelength (nm)

TABLE VI : Relative front (R_f) of dansylated amino acids in thin layer chromatography (TLC) on polyamide sheets

Dansyl Amino acid	Solvent systems			
	Formic acid (1.5%)	Benzene: Acetic acid (9:1 V/V)	Ethylacetate: Acetic acid: Methanol (20:1:1 V/V)	Chloroform : Tertiary butanol: Acetic acid (6:3:1)
Alanine	0.5744	0.6666	0.9568	0.9024
Arginine	-	0.5777	0.4677	0.1120
Asparagine	0.5957	0.7674	0.6581	0.8290
Aspartic acid	0.3133	0.2325	0.7854	0.0800
Glutamic acid	0.5319	0.3333	0.6888	0.7300
Glutamine	0.8297	0.5185	-	0.9268
Glycine	0.5595	0.4666	0.9555	0.6500
Isoleucine	0.2574	0.9532	0.9772	0.9268
Leucine	0.2340	0.6888	0.8444	0.9024
Lysine	0.8162	0.7333	0.4545	0.2350
Methionine	0.2446	0.7555	0.9333	0.2350
Phenylalanine	0.2063	0.8000	0.9333	0.8000
Proline	0.4141	0.9330	0.5444	0.9020
Serine	0.7680	0.2555	0.0412	0.3260
Threonine	0.7659	0.2222	0.7961	0.8520
Tryptophan	0.1276	0.4222	0.8880	0.7500
Tyrosine	0.5319	0.2000	0.8280	-
Valine	0.3893	0.7111	0.9355	0.9630

TABLE VII : Identification of the NH2- and COOH-terminal amino acid residues of buffalo kidney cathepsin B from their corresponding R_f Values on TLC.

	Solvent Systems				
Dansylated Amino acid	Formic acid (1.5%)	Benzene : Acetic acid (9:1 V/V)	Ethylacetate: Acetic acid : (20:1:1 V/V)	Chloform : Tertiary butanol : Acetic acid (6:3:1 V/V)	Result
NH_2-terminal	0.5630	0.666	0.966	-	Ala
COOH-terminal	0.7500	0.222	0.785	0.852	Thr

Takahashi **et al.,** 1984a, 1986; Ritonja **et al.,** 1985 Meloun **et al.,** 1988; Ahmad **et al.,** 1990) where leucine has been suggested as the amino terminal residue.

Determination of COOH-terminal amino acid of buffalo kidney cathepsin B was done according to the method of Narita (1970). The TLC analysis of the released amino acid from cathepsin B on its enzymatic digestion with carboxypeptidase released only one amino acid having the R_f values summarized in table VII. Comparison of the R_f values of the released amino acid with that of standard dansylated amino acid (Tab. VI) showed the released COOH- terminal amino acid to be threonine. This is in accordance with the results reported from other laboratories for cathepsin B from other sources i.e. bovine spleen (Meloun **et al.,** 1988), rat liver (Takio **et al.,** 1983), human liver (Ritonja **et al.,** 1985) and buffalo spleen (Ahmad **et al.,** 1990).

b. Determination of free sulfhydryl groups :

The number of free sulfhydryl groups in cathepsin B was estimated by the method of Ellman (1959). A total of 0.6 and 1.6 moles of thiol groups per mole of cathepsin B were detected under native and denaturing conditions respectively. The result thus showed the presence of more than one free -SH groups which might be partially buried under native conditions (Otto,1970; Takio **et al.,** 1983; Baudys **et al.,** 1991; Musil **et al.,** 1991; Sumiya **et al.,** 1992).

c. Determination of total carbohydrate content :

Determination of the total carbohydrate content of the enzyme was done according to the method of DuBois **et al.,**(1956). Result showed that the buffalo kidney cathepsin B contained about 3.6% carbohydrate as expressed in terms of D-glucose as the standard. This result showed that the kidney enzyme contains relatively lower amount of carbohydrates than its counterpart from the buffalo spleen (Ahmad **et al.,** 1989), bovine and porcine liver and spleen sources (Takahashi **et al.,** 1984a,b; 1986).

d. Amino acid analyses :

Amino acid composition of the enzyme (purified cathepsin B) was studied using the high performance liquid chromatography (**HPLC**) on a Na^+ column. The results are summarized in table VIII. Total tryptophan content of the enzyme was estimated separately using colourimatric method of Spies and Chambers (1949).

Given in table VIII is the amino acid composition of cathepsin B from buffalo kidney. Result showed that the buffalo kidney cathepsin B differs significantly from its counterparts from bovine, human and rat in respect to serine.

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

1. Effect of temperature :

Purified cathepsin B from buffalo kidney had been incubated at varying temperatures ranging from 20°C to about 80°C for two hours and the stability was ascertained after measuring the residual activity against BANA as measured at 37°C by standard

TABLE VIII : Amino acid composition of cathepsin B by HPLC

Amino acid (Mol/Mol Protein)	Bovine (Spleen) [a]	Human (liver) [a]	Rat (liver) [a]	Porcine (spleen) [b]	Buffalo (kidney) [c]
Ala (A)	11	11	14	14	17
Cys (C)	16	14	14	14	14
Asp (D)	11	14	12	25	17
Glu (E)	20	17	17	24	28
Phe (F)	05	08	08	09	09
Gly (G)	33	30	33	33	24
His (H)	08	08	08	06	09
Ile (I)	14	14	15	13	14
Lys (K)	11	10	08	13	10
Leu (L)	07	09	09	11	07
Met (M)	03	04	03	03	04
Asn (N)	14	09	14	d	d
Pro (P)	13	15	12	14	12
Gln (Q)	04	05	05	d	d
Arg (R)	08	09	09	10	09
Ser (S)	21	20	21	21	11
Thr (T)	08	13	12	11	08
Val (V)	14	16	14	14	16
Try (W)	08	08	07	ND	08
Tyr (Y)	10	11	11	11	10

a. Baudys **et al.**, (1991)
b. Takahashi **et al.**, (1986)
c. Determined in this study (Calculated assuming Mol. wt. to be 26 kDa.)
d. Determined as Glu and Asp respectively.
ND. Not determined

procedure described in method section (Pag.45). Figure (21) shows the thermal stability of cathepsin B at varying temperatures. As evident from the figure, buffalo kidney cathepsin B retains its activity well beyond 40°C but below 45°C. At higher temperatures the enzyme is irreversibly inactivated. Figure (22) shows the time dependence of thermal stability of the enzyme. It was found that the enzyme lost its stability and activity rapidly above 48°C.

Temperature has significant effect on the activity of cathepsin B. A bell shaped activity- temperature profile appears to be the characteristic of cathepsin B. The enzyme showed appreciable activity well beyond 42°C with maximal activity at 40°C. The loss of activity was very sharp and rapid beyond 44°C and followed an irreversible path. The loss in activity was, however, reversible below 37°C .

2. Effect of pH on the activity and stability :

The enzyme was very stable between pH 4.6-7.2, with maximal stability at pH 6.8. The enzyme however was highly unstable below pH 2.0 and above pH 7.0.

Cathepsin B from buffalo kidney was found to be active only on a narrow pH zone of 6.0-7.5, having the optimal activity at pH 6.8 against synthetic substrates (Fig.23). This result is consistent with those reported by previous workers (Mason **et al.**, 1986; Agarwal **et al.**, 1987b; Ahmad **et al.**, 1988; Kirschke **et al.**, 1989; Khouri **et al.**, 1991; Hasnain **et al.**, 1992 McDonald **et al.**,

Fig. 21. Effect of temperature on the activity and stability of buffalo kidney cathepsin B.

Appropriate amount of enzyme was exposed to various temperatures for 2.0 hours followed by measurement of its residual activity at 37°C in 20 mM phosphate buffer, pH 6.5, (⊙——⊙). The same buffer was used for assay of residual activity at different temperatures (□——□).

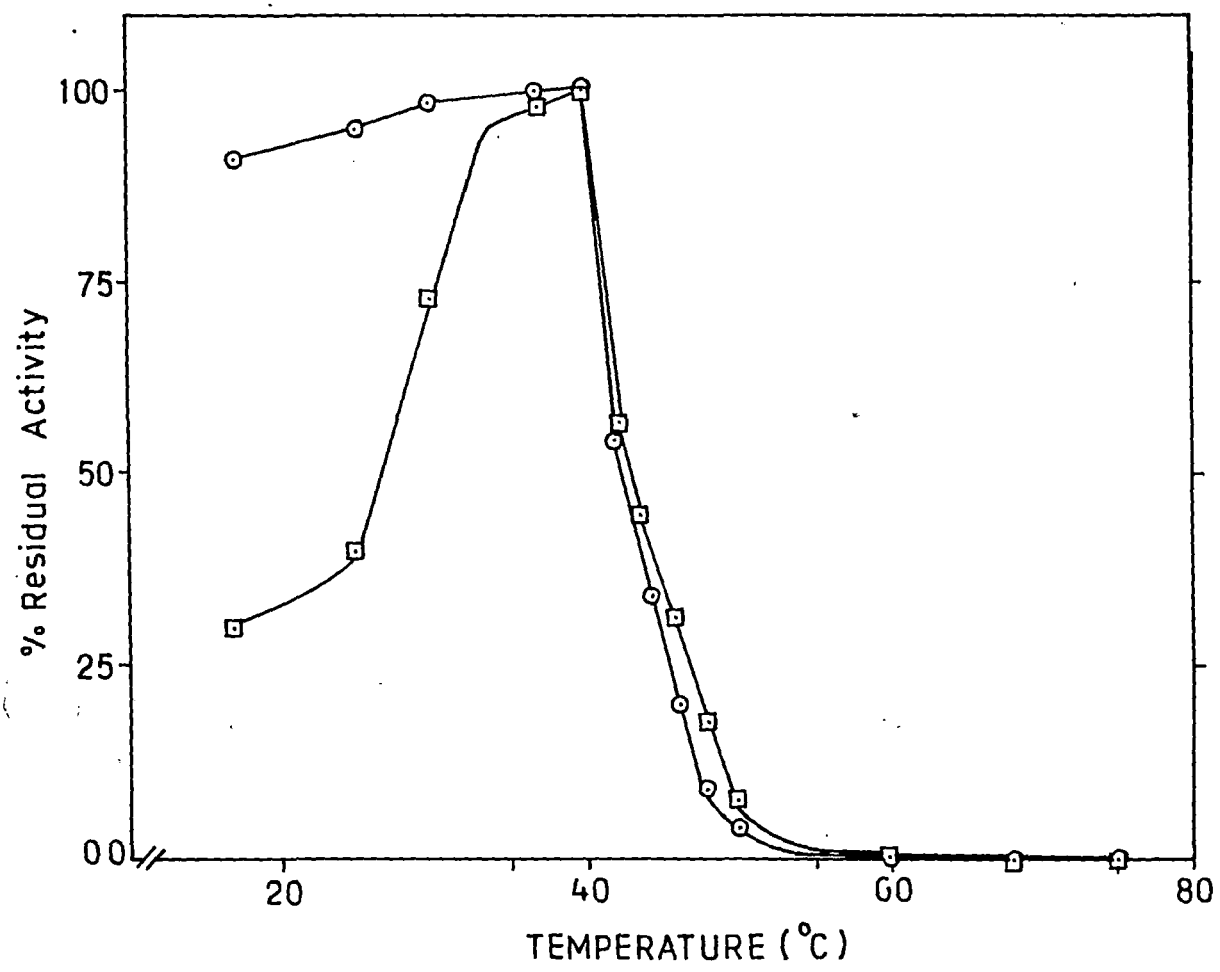
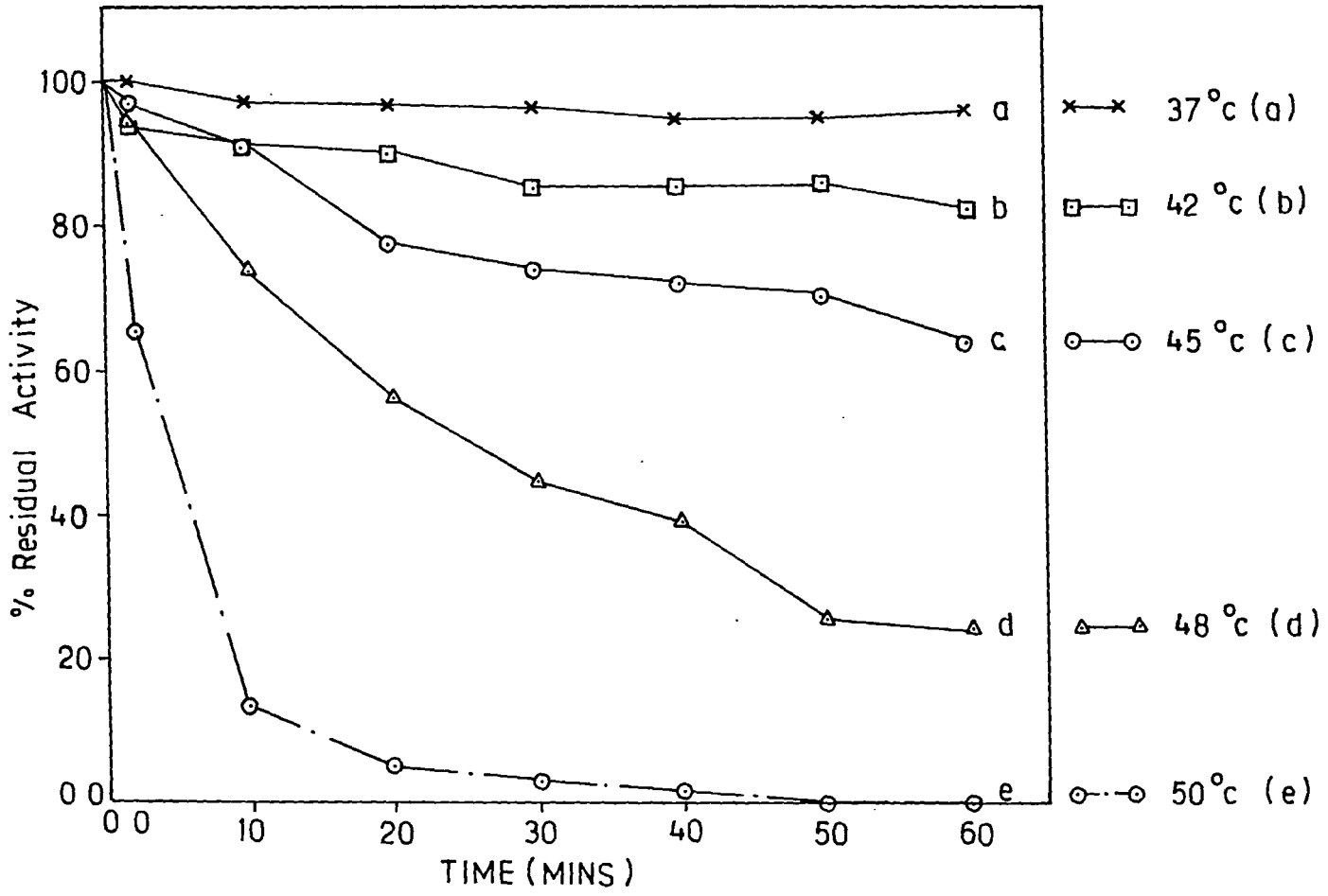
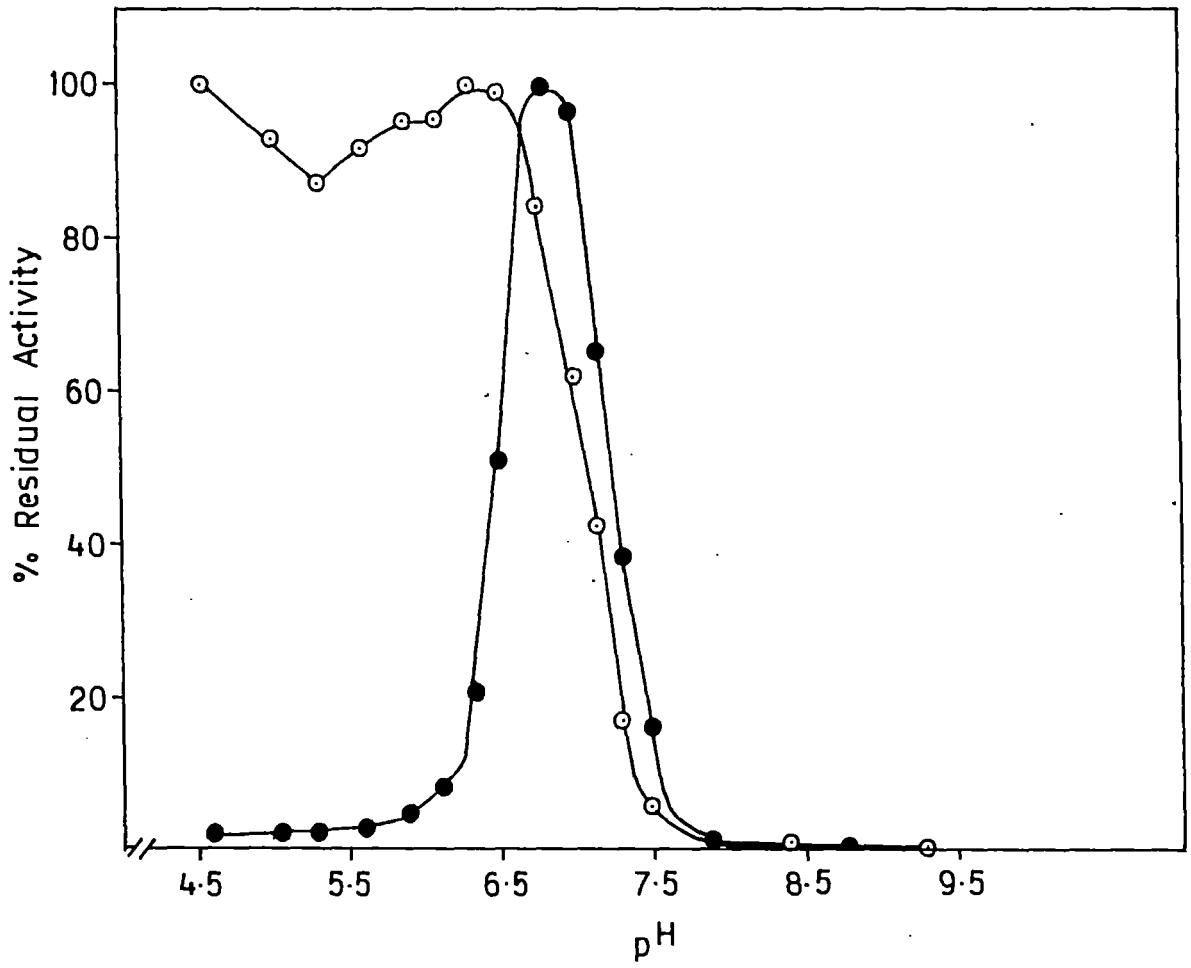

100
75
50
25
00
% Residual Activity
20
40
60
80
TEMPERATURE (°C)

Fig. 22. Time dependence of thermal stability of buffalo kidney cathepsin B
Appropriate amount of enzyme was exposed to different temperatures followed by the measurement of its residual activity at 37°C.



KINETICS OF THERMAL STABILITY OF CATHEPSIN-B

Fig. 23. Effect of pH on the activity and stability of buffalo kidney cathepsin B.
Appropriate amount of enzyme was exposed to various pH conditions for 1 hour prior to the measurement of the residual activity at pH 6.5 in 20 mM phosphate buffer at 37°C (⊙——⊙).
The same buffer was used for the assay of enzymatic activity of at different pHs (●——●).


100
80
60
40
20
% Residual Activity
4·5
5·5
6·5
7·5
8·5
9·5
pH

1993; Sumner **et al.,** 1993). The loss of activity the alkaline pH was found to be irreversible.

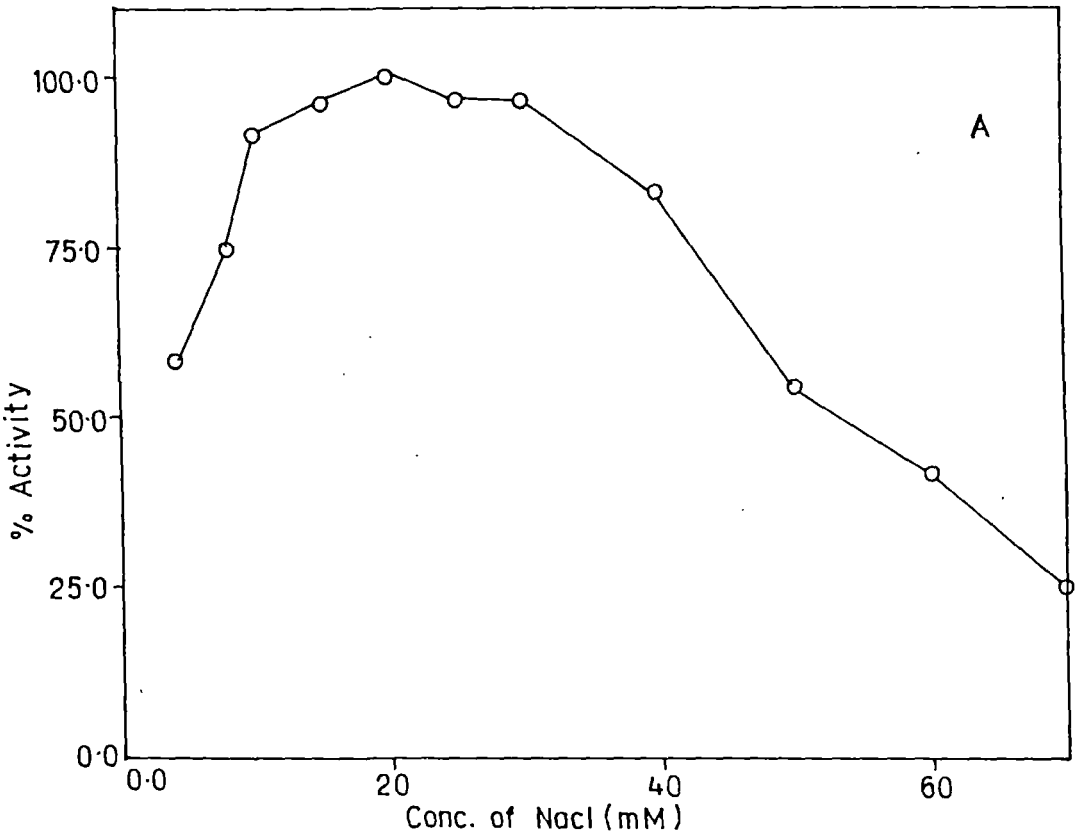
3. Effect of ionic strength and salt concentration :

Purified cathepsin B from buffalo kidney was exposed to varying concentrations of buffer (0.2-80 mM) for about one hour prior to the enzymatic assay at the respective concentrations of phosphate buffer at pH 6.5. Buffer concentration exert a profound effect on the activity of cathepsin B (Fig.24) The enzyme was found to be optimally active between 10-30 mM of buffer, which incidentally falls well within the physiological ionic strength of cellular fluids. There was significant loss of activity at higher ionic strengths, restricting the suitable enzymatic assay zone between 10-30 mM buffer concentrations (Khan **et al.,** 1987; Agarwal **et al.,** 1987a).

4. Effect of Gamma irradiation :

0.2 ml aliquotes of cathepsin B solution in 20 mM phosphate buffer were exposed to different doses of gamma (γ) ray (Co^{60}) followed by the assay of residual activity in the usual manner. The BANA lyase activity of the irradiated enzyme was assayed colourimetrically. Fig. 25 shows the effect of gamma irradiation on the activity of the enzyme. At very low radiation doses (2-20 Gy), the enzyme showed appreciable activity with optimal activity at 8 Gy. At higher radiation dose the enzyme lost its activity significantly. On comparison we found that the enzyme from buffalo kidney is much more sensitive to γ-irradiation than its coun-

Fig. 24. Dependence of cathepsin B activity on salt concentration and buffer strength.
Appropriate amount of enzyme in 2 mM phosphate buffer was exposed to varying concentrations of salts (A) and buffer (B) prior to measurement of catheptic activity in 20 mM phosphate buffer at 37°C.


100·0
75·0
50·0
25·0
0·0
% Activity
A
0·0
20
40
60
Conc. of Nacl (mM)

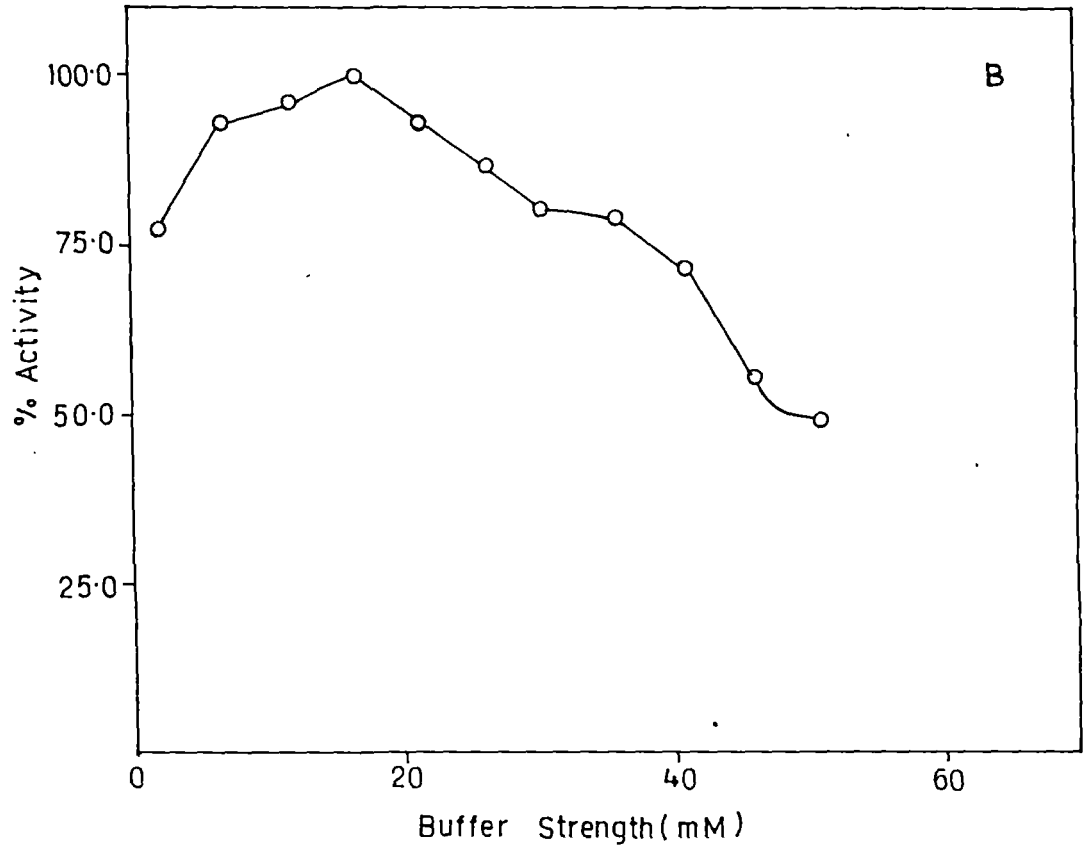
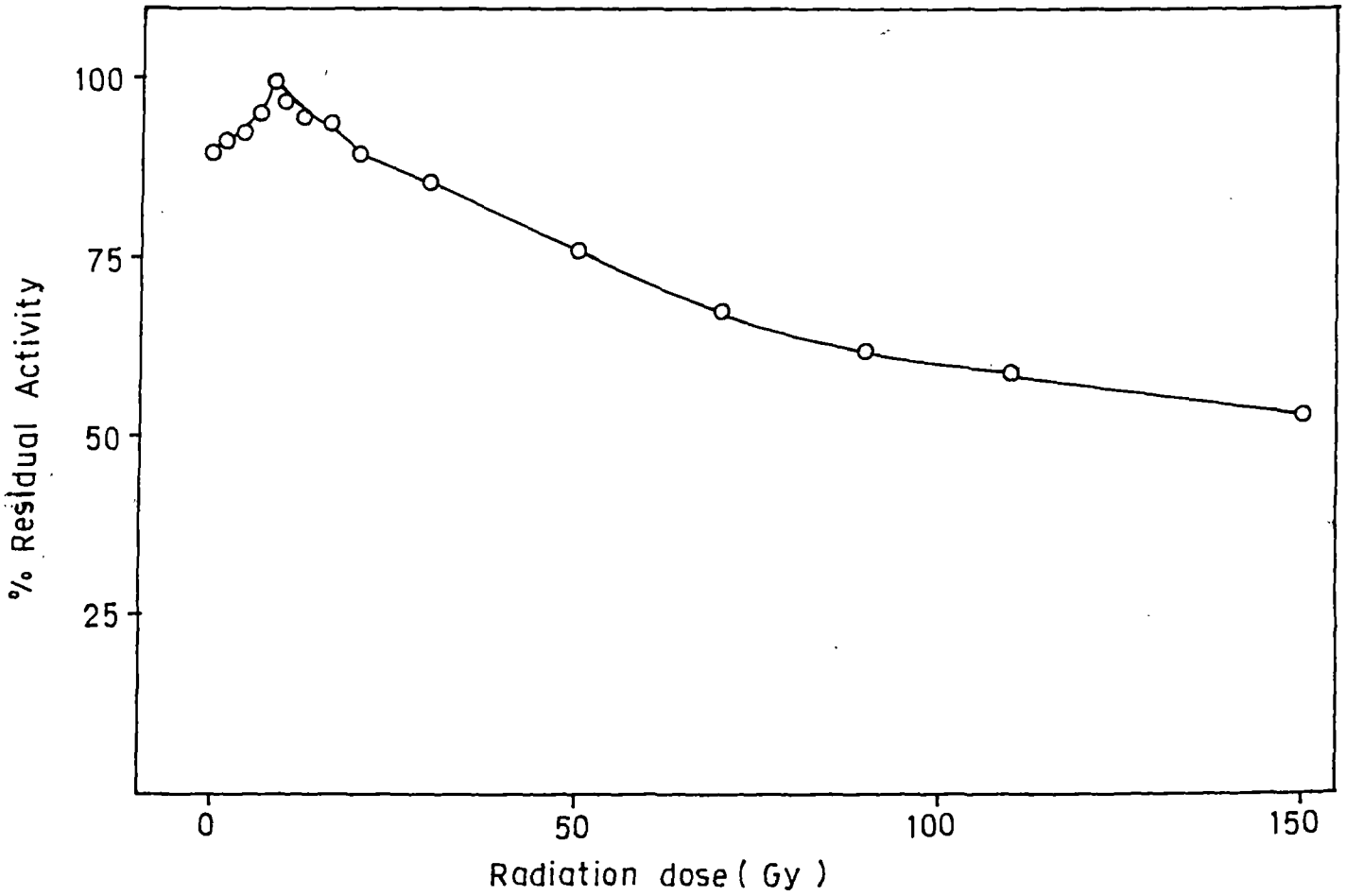

100·0
75·0
50·0
25·0
% Activity
B
0
20
40
60
Buffer Strength (mM)

Fig.25. Effect of gamma radiation on the activity of buffalo kidney cathepsin B .
0.5 ml of enzyme solution (containing fixed amount of enzyme) in 20 mM phosphate buffer, pH 6.5, was exposed to various doses of gamma irradiation followed by the assay of enzymatic activity at 37°C.


100
75
50
25
% Residual Activity
0
50
100
150
Radiation dose (Gy)

terpart from spleen (Ahmad **et al.**,1988) or goat spleen (Agarwal **et al.**, 1987b).

D. Effect of reducing agents :

Effect of various reducing agents such as cysteine base, 2-mercaptoethanol, dithiothreitol (DTT), cysteamine-HCl etc. on kidney cathepsin B was studied after incubating the enzyme with these compounds followed by measurement of their activity colourimetrically in the usual manner. As evident from the results summarised in the table IX, the enzyme activity went up with increasing concentration of reducing agents up to certain level. DTT was found to be the most effective and cysteamine-HCl the least effective (Evans **et al.**, 1983). At higher concentrations of the reducing agents, there was progressive loss of cathepsin B activity. This verifies the classification of the enzyme among the cysteine proteinases which require the exogenous source of thiol groups to express its optimal activity. For most enzymatic assay 2-mercaptoethanol (2 mM) was used because of its higher half life as compared to others (Agarwal **et al.**, 1987a).

E. Effect of thiol blocking compounds :

The effect of heavy metal compounds like $CdCl_2$, $HgCl_2$ and $ZnSO_4$ and thiol blocking reagents such as parahydroxy mercuric benzoic acids (pHMB), Iodoacetamide and iodoacetic acid on the activity of buffalo kidney cathepsin B was studied at different concentration of the effecters ranging from 0-1.0 mM in the usual manner and the results thus obtained have been summarised in

TABLE IX : Effect of reducing compounds on the activity of buffalo kidney cathepsin B

Sl. No.	Conc. (mM)	% Residual activity against			
		2-mercapto-ethanol	L-cysteine	DTT	Cysteamine-HCl
1.	0.0	0.60	0.60	0.60	0.60
2.	1.0	20.54	37.67	48.63	17.80
3.	2.0	30.82	50 68	56.16	19.49
4.	4.0	46.57	55.47	74.65	21.23
5.	6.0	53.42	58.90	100.00	21.23
6.	8.0	60.27	58.21	92.46	21.23
7.	10.0	65.06	54.10	54.24	31.50
8.	15.0	73.97	31.50	46.57	38.35

Enzyme activity obtained in the presence of 6.0 mM DTT was taken as 100%.

TABLE X : Effect of thiol blocking compounds on the activity of buffalo kidney cathepsin B

Conc. (mM)	% Residual activity against					
	$CdCl_2$	$HgCl_2$	$ZnSO_4$	Iodoacet-amide	Iodoacetic acid	p-hydroxy mercuric benzoate
0.00	100.00	100.00	100.00	100.00	100.00	100.00
0.05	99.80	95.00	98.00	40.64	13.04	100.00
0.10	99.00	90.25	97.50	25.00	3.34	99.88
0.15	98.60	85.00	95.00	13.04	1.50	99.50
0.20	98.55	78.50	92.50	9.69	1.00	99.27
0.40	97.34	61.59	71.65	4.68	0.33	87.43
0.60	93.23	36.23	50.00	3.34	0.00	82.36
0.80	88.16	13.04	35.50	2.00	0.00	81.64
1.00	83.81	3.62	20.75	2.00	0.00	64.97

table X. Enzyme activity in absence of these effecters was taken as 100%. As evident from the result, mercuric and zinc compound acted as the powerful inhibitors of the kidney enzyme as compared to cadmium (McKim **et al.,** 1992). Buffalo kidney cathepsin B was also found to be very sensitive to iodoacetic acid and virtually no activity was left at a concentration as low as 5 mM. The result also confirmed the presence of a thiol group at active site of the enzyme (Takahashi **et al.,** 1986).

F. Effect of peptidyl inhibitors :

Effect of a few peptidyl inhibitors such as E-64, leupeptin, antipain, chymostatin and pepstatin A on the activity of the purified cathepsin B was studied after exposing the enzyme to varying concentration of the inhibitors for 2 mins. prior to the measurement of residual enzyme activity in the usual manner using BANA and Z- Arg-Arg-MCA as substrates. As evident from table XI, of all the inhibitors used, E-64 proved to be the most potent followed by leupeptin. This result is in consistence with what has been reported by earlier workers for other sources (Medhi **et al.,** 1991; Rifkin **et al.,** 1991; Gour-Salin **et al.,** 1993; Sumiya **et al.,** 1992; Walker **et al.,** 1993; Montenez **et al.,** 1994). Pepstatin on the other hand, showed no appreciable stimulatory or inhibitory effect. This study showed that the enzyme preparation to be pure cathepsin B. Since pepstatin inhibits cathepsin D and cathepsin H is less sensitive to leupeptin, the probability of contamination of the enzyme (cathepsin B) preparation with ca-

TABLE XI : Effect of peptidyl inhibitors on buffalo kidney cathepsin B activity

Conc. (μM)	% Residual activity against			
	E-64	Leupeptin	Antipain	Pepstatin
0.00	100.00	100.00	100.00	100.00
0.03	52.18	60.05	89.90	101.21
0.06	33.49	37.93	65.27	100.00
0.15	14.80	16.25	32.93	97.82
0.30	7.76	9.36	16.16	94.66
0.60	0.05	5.17	7.18	101.21
0.90	0.00	4.18	3.59	104.85
1.20	0.00	2.95	2.99	97.09
1.50	0.00	2.46	2.39	89.80

thepsin D & H was negligible (Tanaka **et al.,** 1984; Oshita **et al.,** 1992; Montenez **et al.,** 1994).

G. Effect of denaturants on the activity of Cathepsin B :

1. Effect of Urea :

In an attempt to investigate the structure- function relationship of the purified kidney enzyme, urea induced inactivation was carried out at 37°C in 20 mM sodium phosphate buffer, pH 6.5 and the result thus obtained has been summarised in figure 26. As evident from the curve, enzyme activity was found to be highly sensitive towards urea and about 50% of the activity was lost at a urea concentration of about 1.0 M. This urea concentration, although higher than those reported from goat enzyme (Agarwal **et al.,** 1988), is well in agreement with the inhibitory urea concentration reported for buffalo spleen enzyme (Ahmad **et al.,** 1989). The reversibility of the inactivated enzyme was also measured after exposing the enzyme at various higher urea concentrations and diluting it to lower urea concentrations. The total recovery of the lost activity was possible when the enzyme was exposed to urea concentrations less than 2.0 M. In contrast, when the enzyme was exposed to the urea concentrations higher than 3.0 M, no activity could be recovered back suggesting the irreversible loss of enzyme activity at those concentrations of urea.

2. Effect of guanidine hydrochloride :

Cathepsin B was incubated with varying concentrations of guanidine hydrochloride for 30 mins. at room temperature and its

Fig. 26. Effect of urea on the buffalo kidney cathepsin B activity
Effect of urea concentration on the activity of cathepsin B (○) was measured at 37°C using BANA as substrate. For reactivation studies, the enzyme was exposed to 3.0 M (□), 2.5 M (△), 2.0 M (×) and 1.0 M (●) urea for 6 hrs. at room temperature. Residual BANA-lyase activity was measured after diluting the urea to lower concentrations. Enzyme activity in absence of urea was taken as 100%.

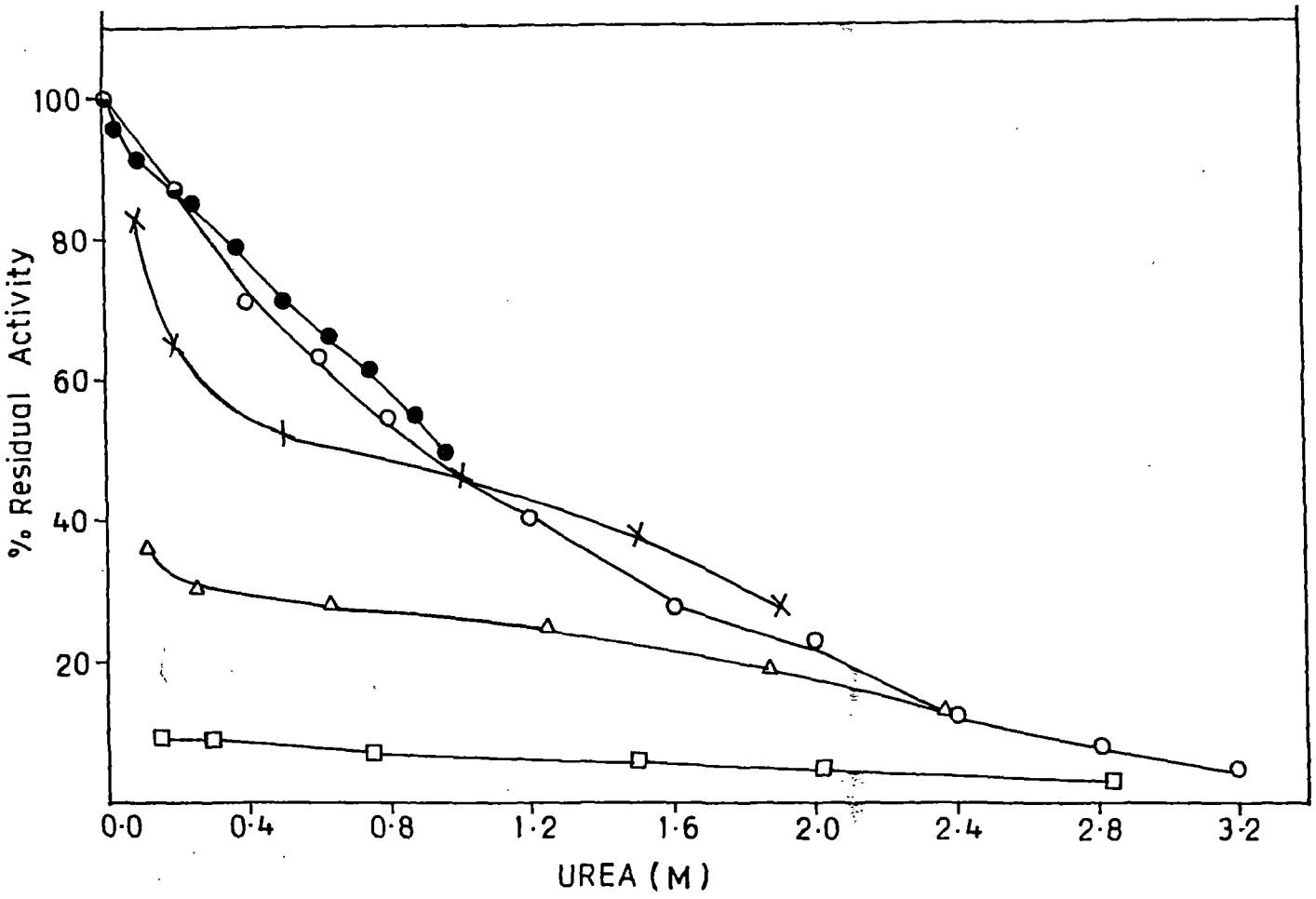
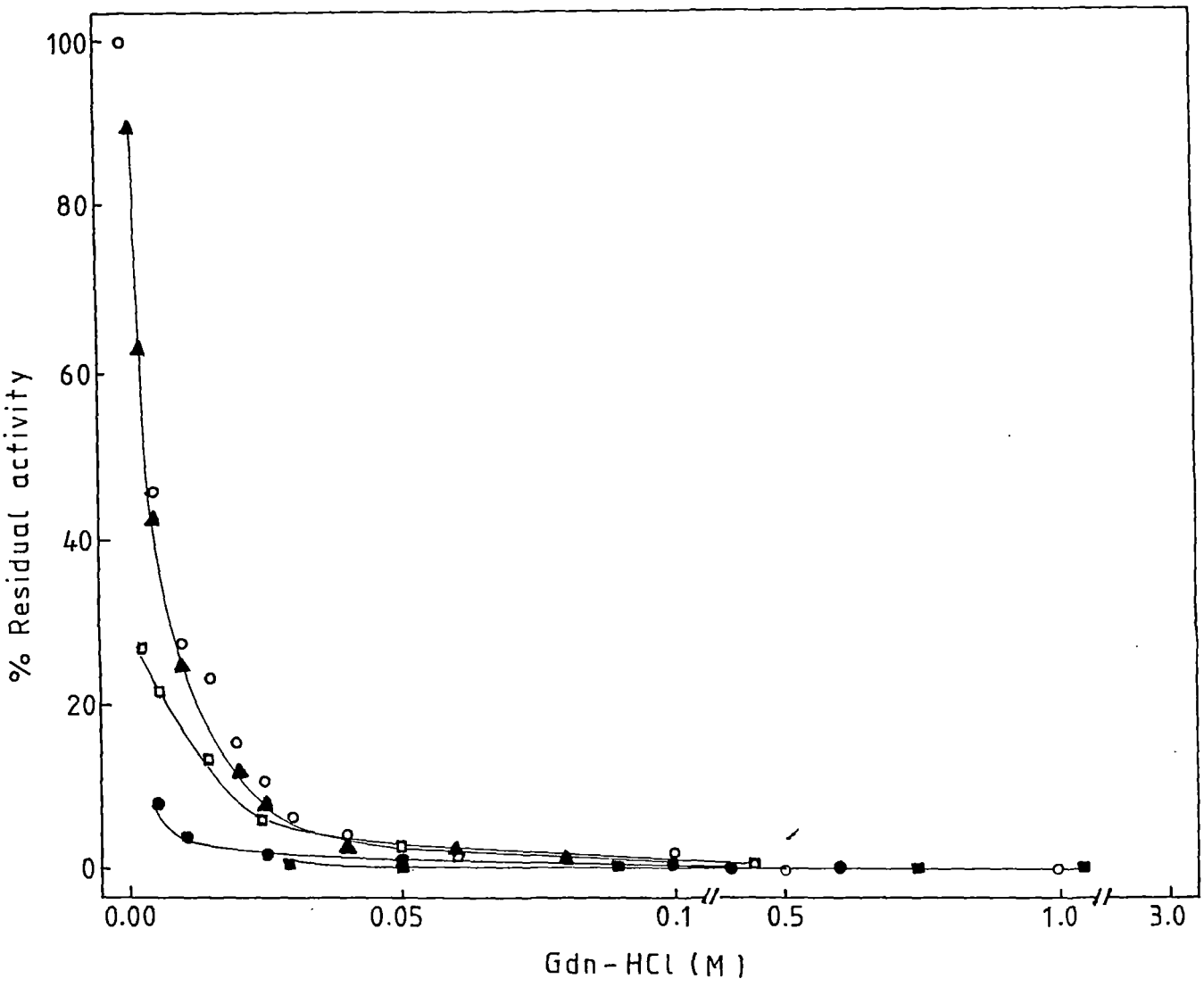

100
80
60
40
20
% Residual Activity
0·0
0·4
0·8
1·2
1·6
2·0
2·4
2·8
3·2
UREA (M)

Fig. 27. Effect of Gdn-HCl on the activity of buffalo kidney cathepsin B.
Dependence of Gdn-HCl concentrations on the activity of cathepsin B (o). For reactivation studies, the enzyme was exposed to 3.0 M (■), 1.0 M (●), 0.5 M (□) and 0.1 M (▲) Gdn- HCl for 30 mins. at 25°C. The residual BANA-lyase activity was measured after diluting the denaturants to lower concentrations. Enzyme activity in absence of Gdn-HCl was taken as 100%.


100
80
60
40
20
0
% Residual activity
0.00
0.05
0.1
0.5
1.0
3.0
Gdn-HCl (M)

residual activity was measured in usual manner. The results thus obtained have been summarised in figure 27. As evident, cathepsin B was rapidly inactivated by guanidine-HCl and the latter is several fold more effective in inactivating the enzyme than urea. More than 50% of the enzyme activity was suppressed at the denaturant concentration as low as 10 mM and no activity was left at 50 mM and above.The loss of activity was reversible when the enzyme was exposed to Gdn-HCl concentration less than 0.5 M, with full recovery at 0.1 M. In contrast, there was irreversible loss of enzyme activity above 2.0 M Gdn-HCl (Khan **et al.,** 1992).

H. Kinetic studies against substrates :

The values of K_m and V_{max} for both synthetic peptide (Fig. 28 & 29) as well as protein substrates (Fig. 30 & 31), were computed using the best fit plot analysis of the data plotted according to method of Lineweaver and Burk (1934). Table XII summarises the results on the kinetic studies of the purified buffalo kidney cathepsin B against various synthetic and protein substrates. The K_m of the enzyme followed the order Z-Phe-Arg-MCA < Z-Arg-Arg-MCA < BAPNA < BANA with the values of 0.0909 mM, 0.166 mM, 1.818 mM and 3.3 mM respectively for these four synthetic substrates.

Although the values of K_m found by us fall well within the range, the V_{max} values of the enzyme vary drastically from the literature values of cathepsin B from other sources (Tchoupe **et al.,** 1991; Fox **et al.,** 1992). The buffalo kidney enzyme had

TABLE XII : Kinetic parameters of buffalo kidney cathepsin B

Sl.No.	Substrate	K_m	V_{max}	
1.	BANA	3.3000 mM	0.38460	units[a]
2.	BAPNA	1.8180 mM	0.05714	-do-
3.	Z-Phe-Arg-MCA	0.0909 mM	1.53840	-do-
4.	Z-Arg-Arg-MCA	0.1660 mM	0.85106	-do-
5.	Casein	6.7790 µM	1.42860×10^3	units[b]
6.	BSA	3.0303 µM	0.41660×10^3	-do-
7.	Haemoglobin (goat)	1.4280 µM	1.25000×10^3	-do-
8.	Haemoglobin (buffalo)	2.1730 µM	0.60600×10^3	-do-

a : µMol/mg/min b : Change of O.D. by 0.01 units/mg/hr.

appreciably higher K_m for BANA than its counterpart from buffalo spleen, but showed close similarity with the liver enzyme. This result may not be surprising since the spleen cells display histolytically much more complexity as compared to the kidney and liver cells. Hence the tissue specificity of the enzyme can not be ruled out altogether (Takio **et al.,** 1983; Takahashi **et al.,** 1984a,b; Bechet **et al.,** 1986: Nishimura **et al.,** 1988; Pagano **et al.,** 1988; Mach **et al.,** 1992).

Cathepsin B has long been demonstrated to be very active against protein substrates like muscle aldolase, glucagon, oxydised B-chain of insulin, collagen, denatured haemoglobin, fibrinogen and fibronectins at acidic pH of about 5.0. Our kinetics results on buffalo kidney cathepsin B showed that the enzyme is very effective against BSA, casein and denatured haemoglobin even at mild acidic range or near neutral pH zone. Goat haemoglobin (K_m= 1.428 μM) proved to be the most effective substrate followed by buffalo haemoglobin (K_m= 2.173 μM), BSA (K_m= 3.0303 μM) and casein (K_m= 6.779 μM). On comparison it was found that the kidney enzyme was less efficient against these protein substrates than its counterpart from the spleen (Ahmad **et al.,** 1990).

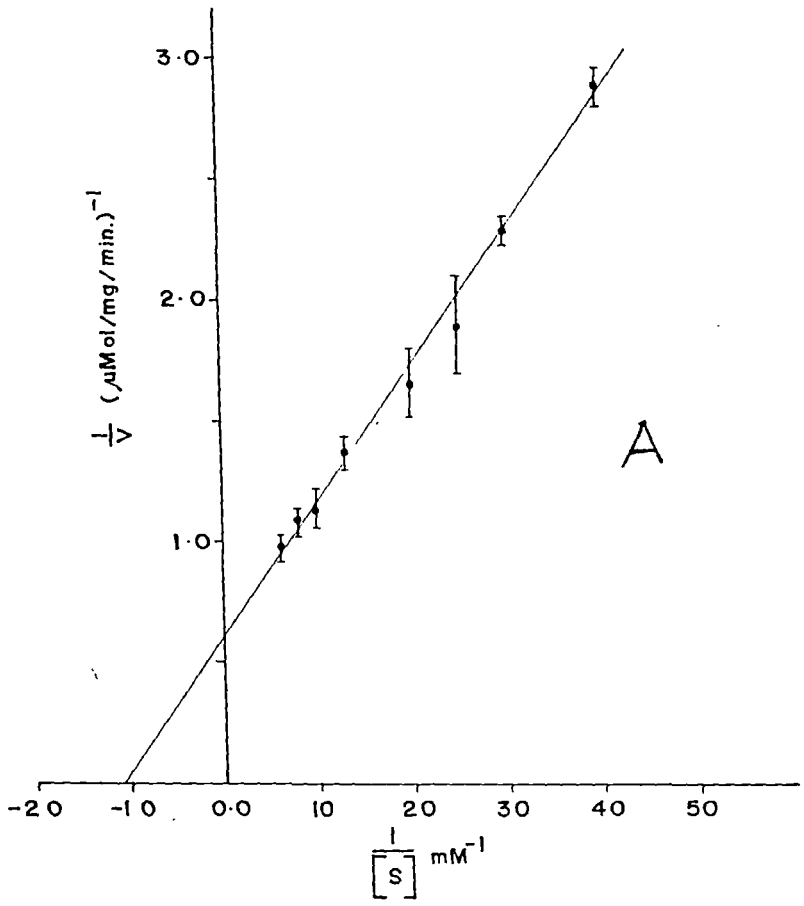
The differential catalytic efficiency of the enzyme from different tissue sources could have been arisen with the need to perform some specialised functions in those tissues (Pagano **et al.,**1988; Mach **et al.,** 1992).

I. Aldolase inactivation studies :

Until recently, aldolase inactivation was considered to be

Fig. 28. Lineweaver Burk plot of buffalo kidney cathepsin B using Z-Phe-Arg-MCA(A) and Z-Arg-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 4 mM EDTA and 8 mM DTT. The bar represents the range of the values of three independent experiments.


3·0
2·0
1·0
$\frac{1}{V}$ (μMol/mg/min.)$^{-1}$
A
-2·0
-1·0
0·0
1·0
2·0
3·0
4·0
5·0
$\frac{1}{[S]}$ mM^{-1}

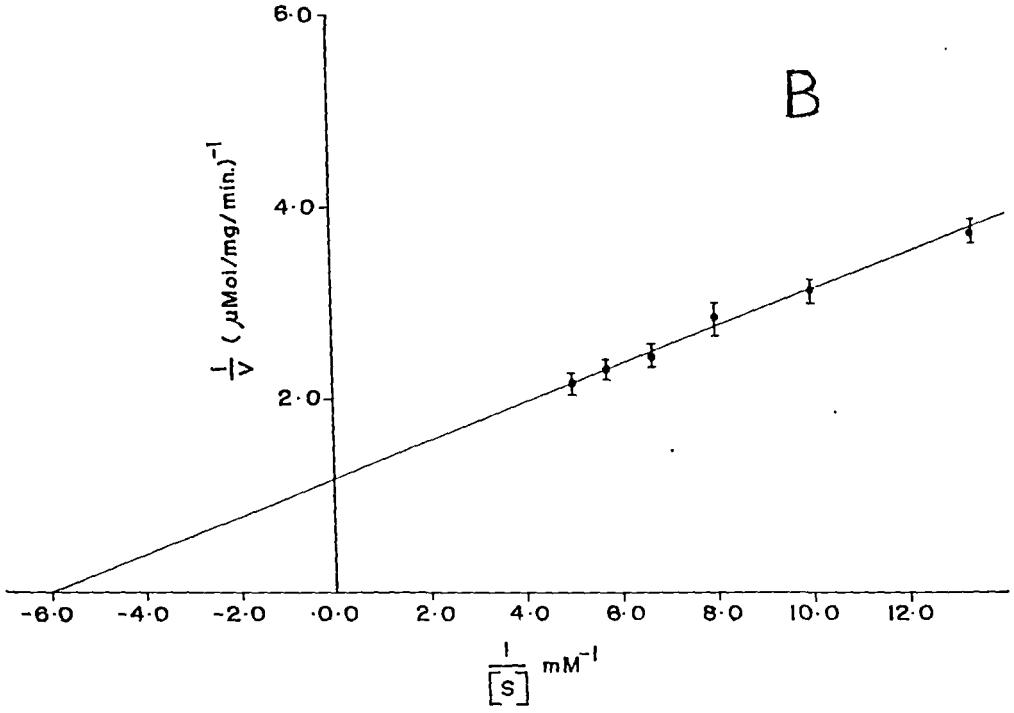
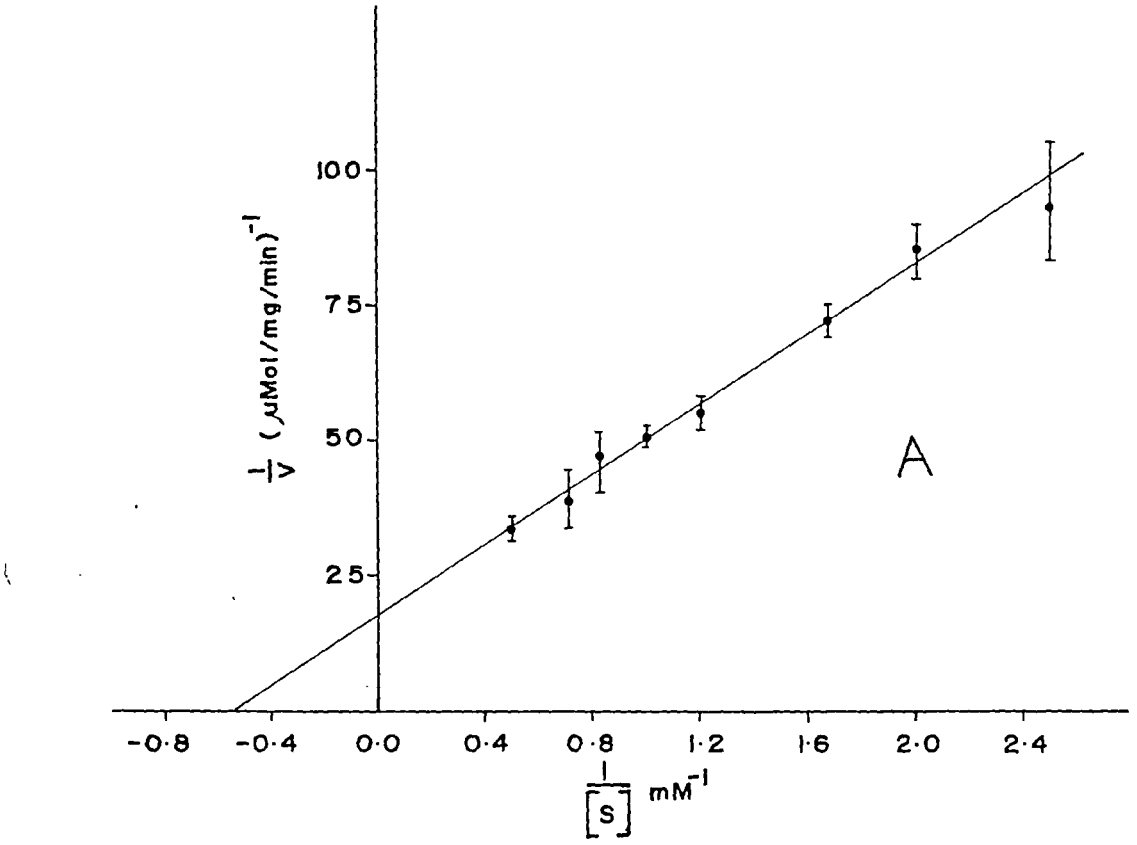

6·0
4·0
2·0
$\frac{1}{V}$ (μMol/mg/min.)$^{-1}$
B
-6·0
-4·0
-2·0
·0·0
2·0
4·0
6·0
8·0
10·0
12·0
$\frac{1}{[S]}$ mM^{-1}

Fig. 29. Lineweaver Burk plot of buffalo kidney cathepsin B using BAPNA (A) and BANA (B) as substrates.

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 range of the values of three independent experiments.


100
75
50
25
$\frac{1}{V}$ (μMol/mg/min)$^{-1}$
A
-0·8
-0·4
0·0
0·4
0·8
1·2
1·6
2·0
2·4
$\frac{1}{[S]}$ mM^{-1}

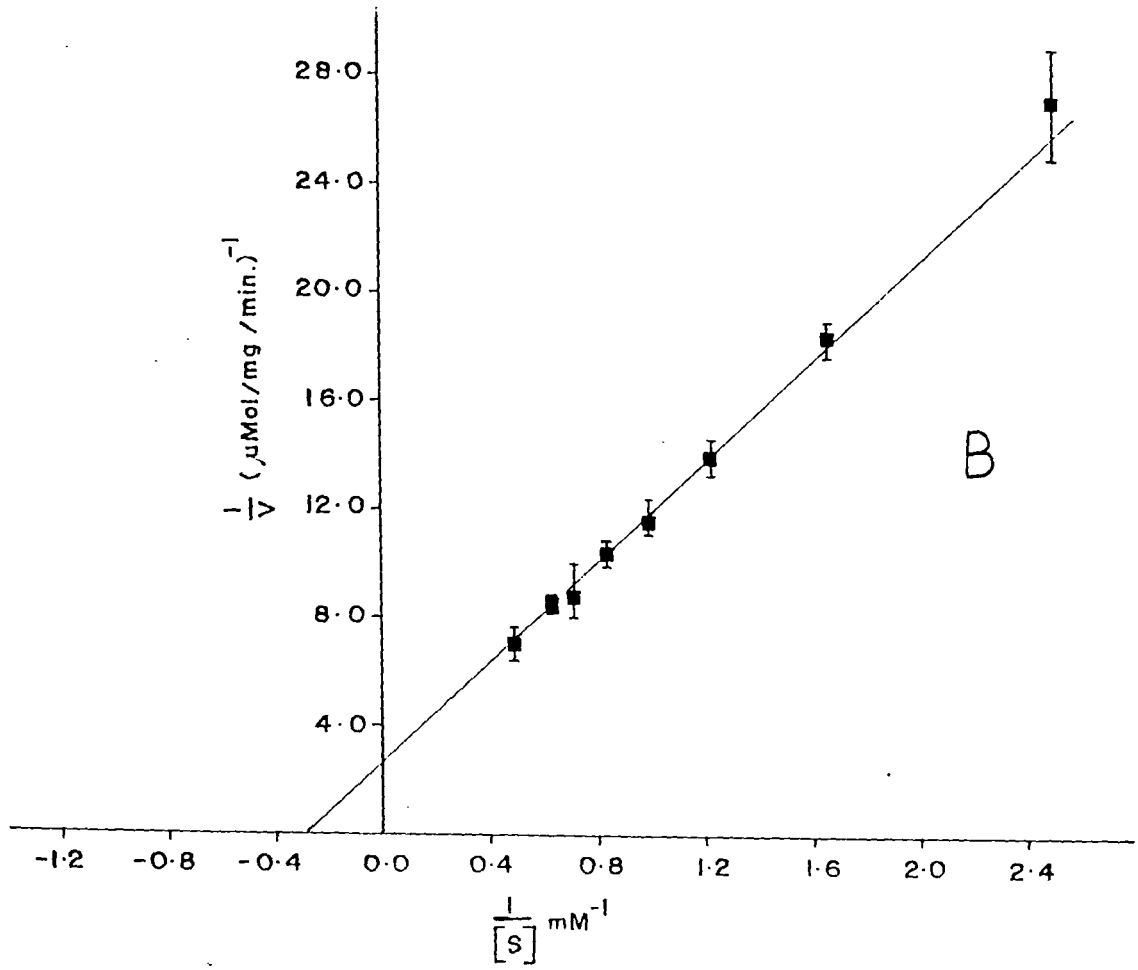
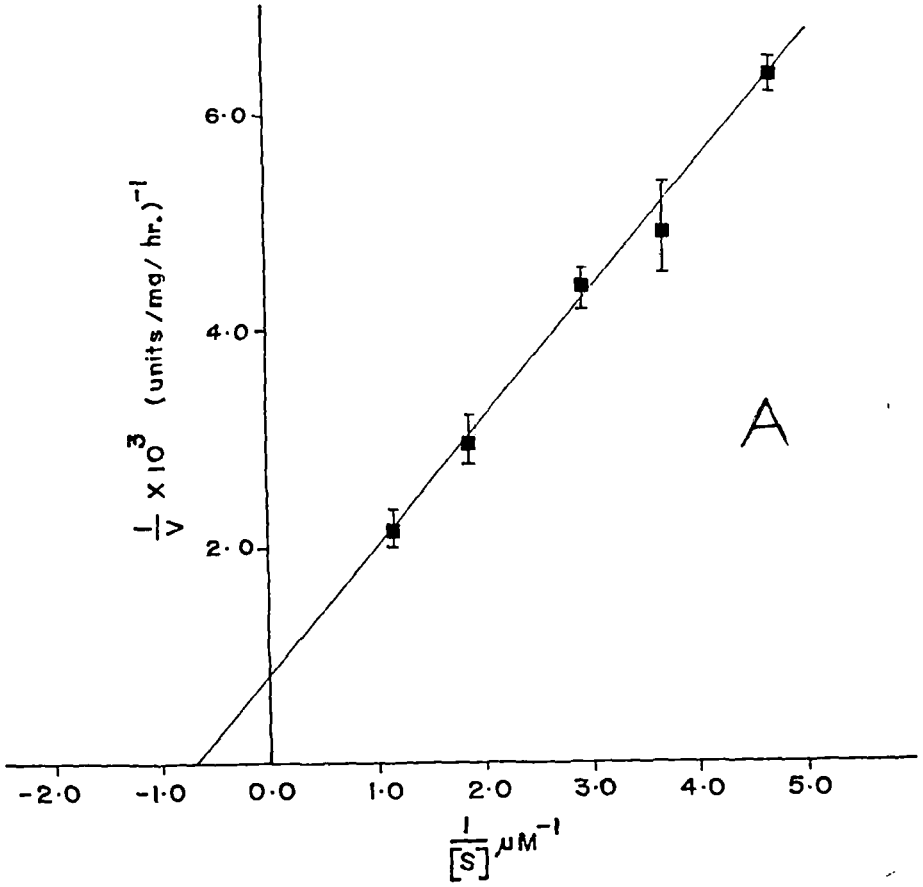

28·0
24·0
20·0
16·0
12·0
8·0
4·0
$\frac{1}{V}$ (μMol/mg/min.)$^{-1}$
B
-1·2
-0·8
-0·4
0·0
0·4
0·8
1·2
1·6
2·0
2·4
$\frac{1}{[S]}$ mM^{-1}

Fig. 30. Lineweaver Burk plot of buffalo kidney cathepsin B using goat (A) and buffalo (B) haemoglobin as substrates.

The experimental details were similar to those described in legend to Fig. 29.


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

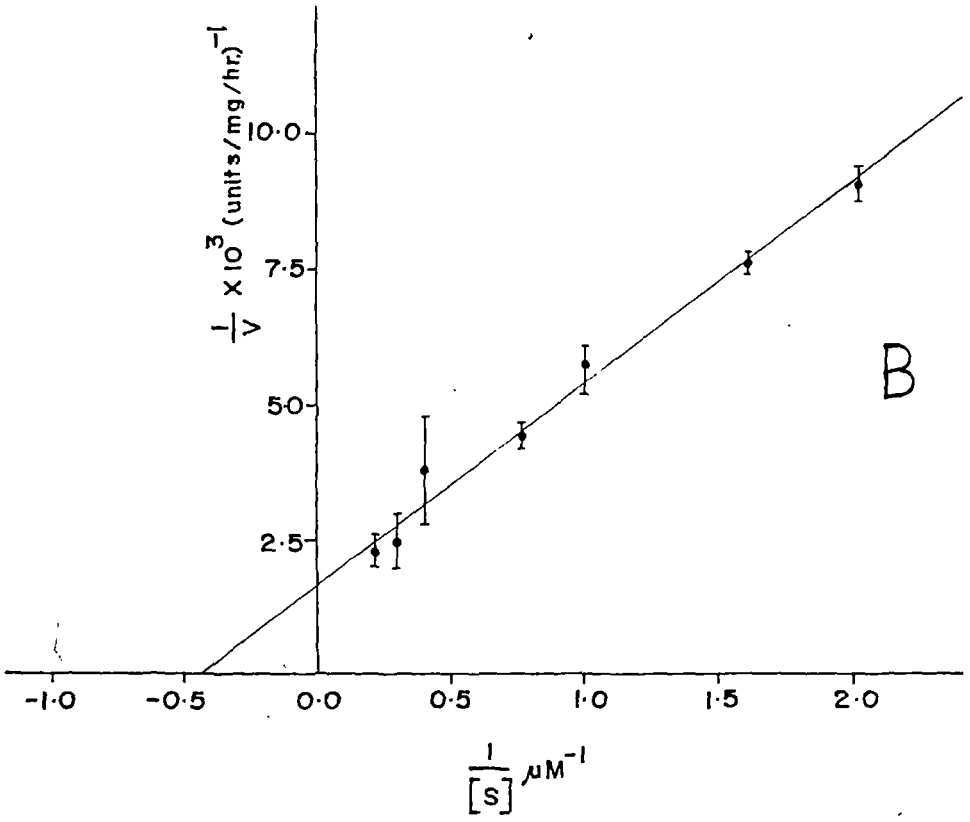
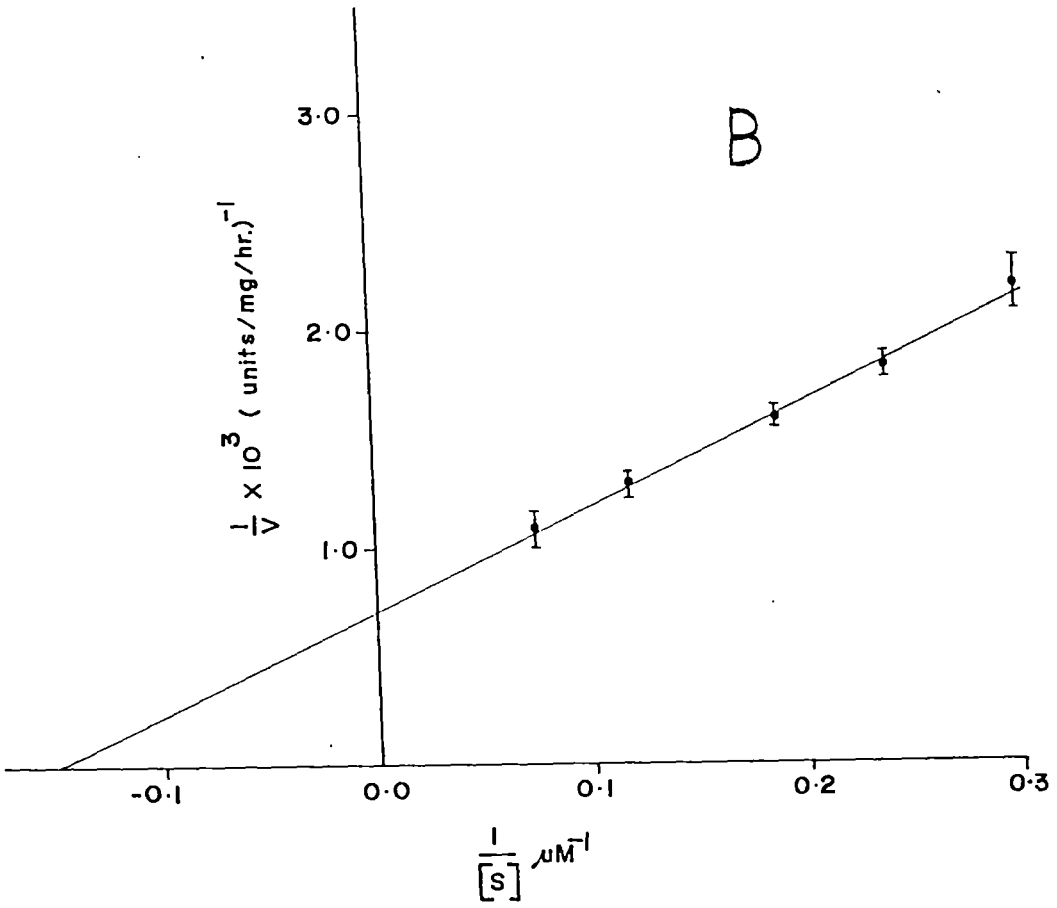
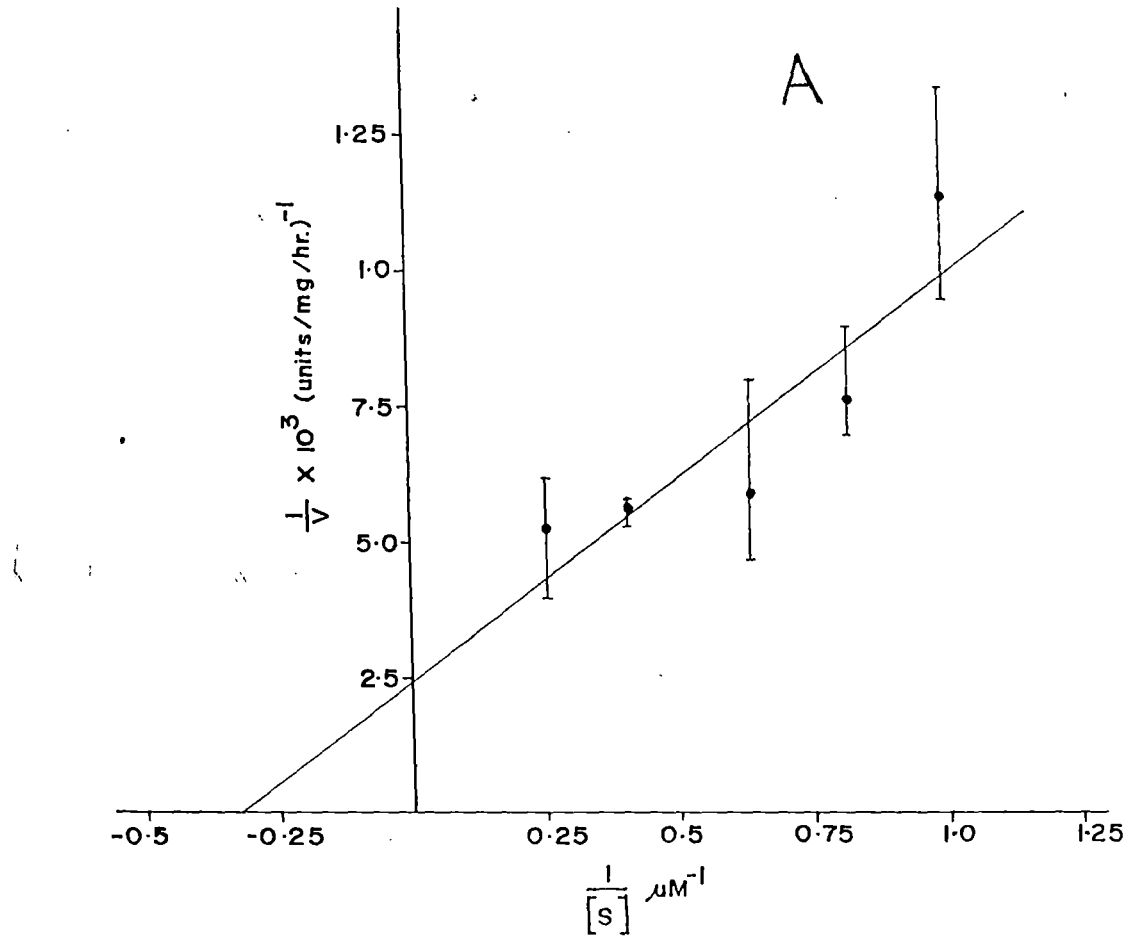

10·0
7·5
5·0
2·5
$\frac{1}{v} \times 10^3$ (units/mg/hr.)$^{-1}$
B
-1·0
-0·5
0·0
0·5
1·0
1·5
2·0
$\frac{1}{[S]}$ μM^{-1}

Fig. 31. Lineweaver Burk plot of buffalo kidney cathepsin B using BSA (A) and bovine milk casein (B) as substrates.

The experimental details were similar to those described in legend to Fig. 29.


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


A
1·25
1·0
7·5
5·0
2·5
$\frac{1}{V} \times 10^3$ (units/mg/hr.)$^{-1}$
-0·5
-0·25
0·25
0·5
0·75
1·0
1·25
$\frac{1}{[S]}$ μM^{-1}

the distinctive characteristics of cathepsin B to differentiate this enzyme from other cathepsins viz., cathepsin H, L, M, S, T etc. Buffalo kidney cathepsin B was incubated with rabbit muscle aldolase (molar ratio 1:50) in 0.1 M sodium phosphate buffer, pH 6.5 containing 2 mM 2-mercaptoethanol. Aliquote of the mixture was used to measure the residual aldolase activity at various intervals of time during the incubation by following the hydrazine method described in the " Worthington manual" (or Sigma procedure No.752). Our results on the purified kidney cathepsin B showed that the enzyme had less affinity for aldolase. Only about 25% inactivation of the aldolase was observed (Fig. 32) as against about 75-80% inhibition reported for the porcine and bovine enzymes (Towatari **et al.**, 1979; Bond **et al.**, 1980; Takahashi **et al.**, 1986).

J. Immunological studies :

The antiserum produced in rabbits against buffalo kidney cathepsin B was found to be specific for cathepsin B (Fig. 33) only and did not cross react with other cathepsins, viz., cathepsin H or L from the same or different sources. However, the antibody could recognise the cathepsin B from bovine tissue sources (Tab. XIII)

To test the structural transformations at alkaline pH or in presence of inhibitors like iodoacetamide and iodoacetic acid, Ouchterlony double immunodiffusion was carried out at pH 6.8 and 8.0, in presence and absence of inhibitors. In both the cases

Fig. 32. Aldolase inactivation by buffalo kidney cathepsin B. Buffalo kidney cathepsin B was incubated with rabbit muscle aldolase (molar ratio 1:50) in 0.1 M sodium phosphate buffer, pH 6.5, containing 2 mM 2-mercaptoethanol for various time intervals. Measurement of residual aldolase activity was done following the hydrazine method as described in Sigma procedure No. 752. (a) Control, (b) Test.

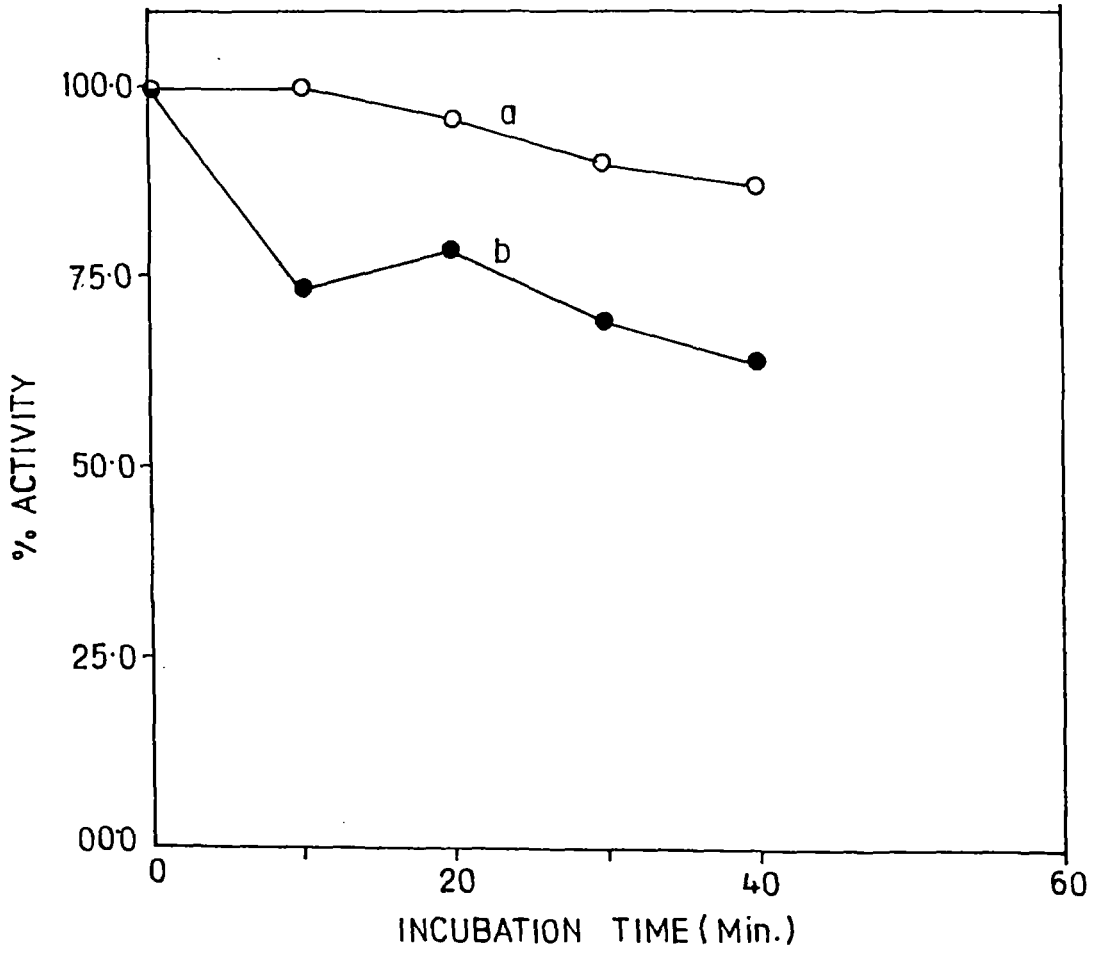

100·0
75·0
50·0
25·0
000
% ACTIVITY
a
b
0
20
40
60
INCUBATION TIME (Min.)

Fig. 33. Ouchterlony double immunodiffusion of purified buffalo kidney cathepsin B with anti-cathepsin B antibody.

About 200 µl of antiserum (Ab) and 50 µl of purified buffalo kidney cathepsin B was applied in the wells and incubated overnight at 37°C in phosphate buffer saline, at pH 7.2.

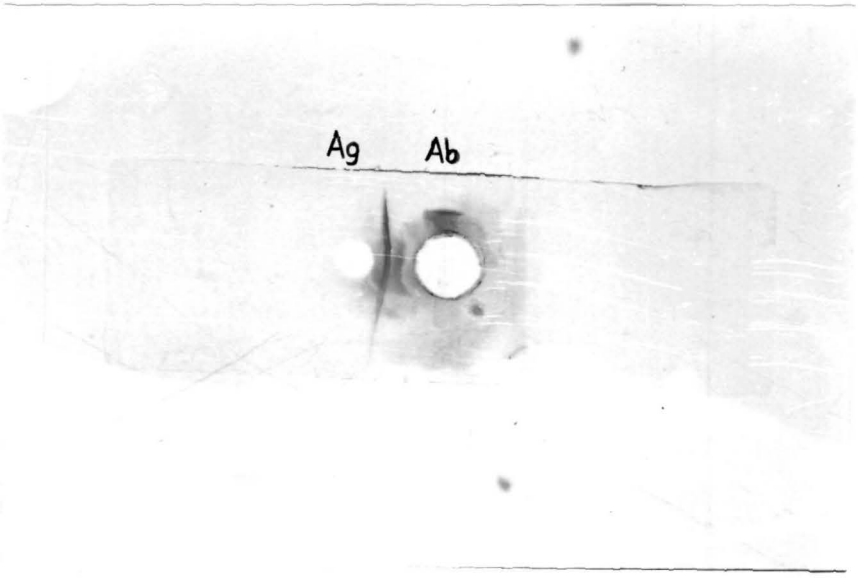

Ag
Ab

TABLE XIII : Reaction of anti-cathepsin B (buffalo kidney) antibody against cathepsins B and H from various sources

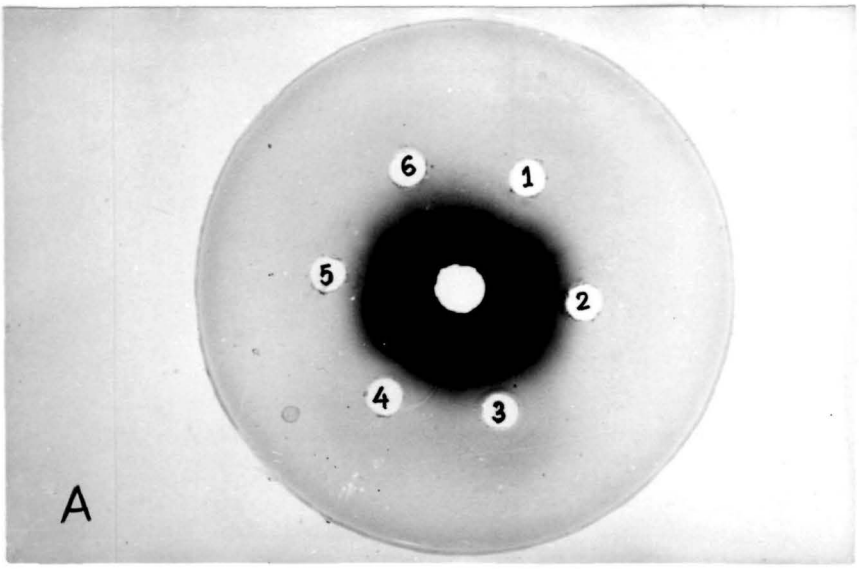
Antigen	Source	Condition	Result
C_B	buffalo kidney	native	+
C_B	buffalo kidney	pH denatured	+
C_B	buffalo spleen	native	+
C_B	bovine spleen	native	+
C_B	goat spleen	native	-
C_H	buffalo kidney	native	-
C_H	buffalo spleen	native	-
C_H	porcine lungs	native	-

C_B : cathepsin B; (+) : cross reacts
C_H : cathepsin H; (-) : does not cross react

Fig. 34. Ouchterlony double immunodiffusion of cathepsins at pH 6.8.

A. The central well contained antiserum raised against buffalo kidney cathepsin B. The peripheral wells starting from 1 clockwise, contained buffalo kidney cathepsin B, porcine lungs cathepsin H, goat spleen cathepsin B, bovine spleen cathepsin H, buffalo kidney cathepsin H and bovine spleen cathepsin B.

B. The central well contained antiserum raised against buffalo kidney cathepsin B. The perpheral wells starting from 1 clockwise, contained buffalo kidney cathepsin B, buffalo spleen cathepsin B, buffalo kidney cathepsin H, porcine lungs cathepsin H, buffalo kidney cathepsin L and bovine spleen cathepsin B.


6
1
5
2
4
3
A

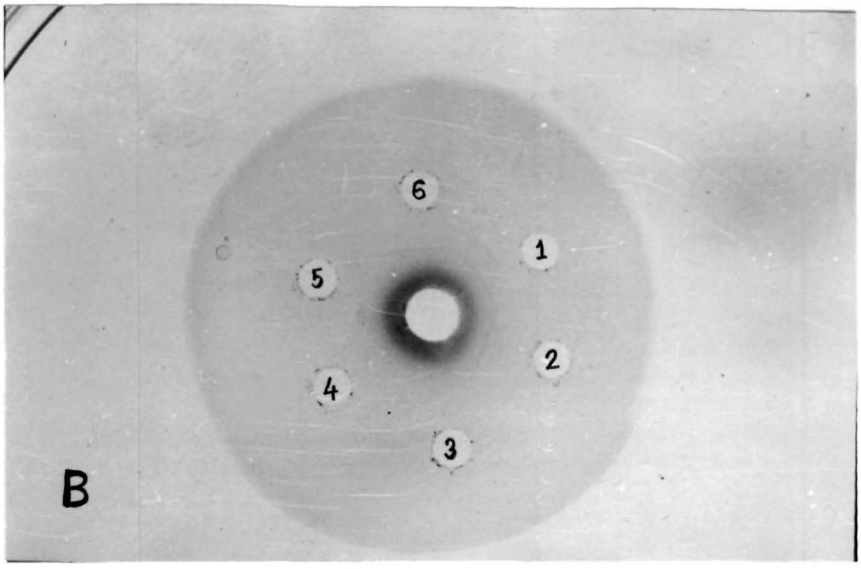
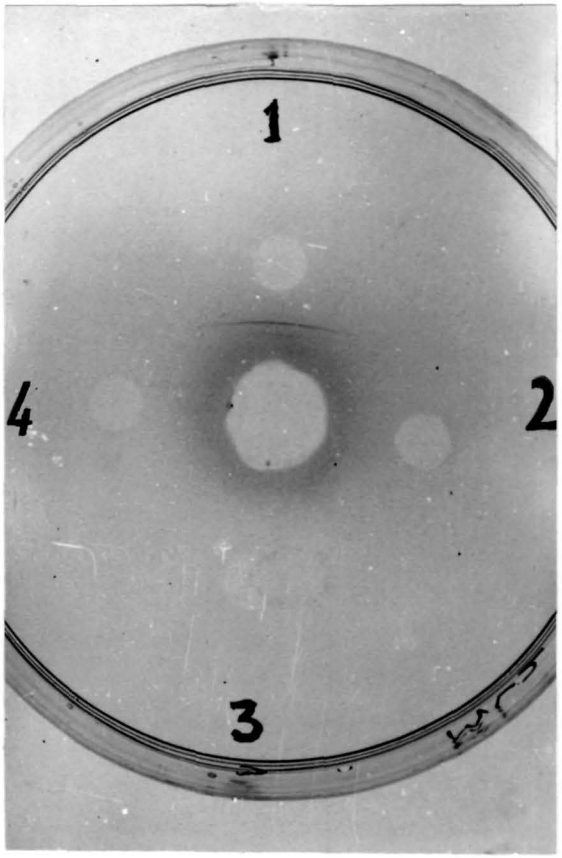

6
1
5
2
4
3
B

Fig. 35. Ouchterlony double immunodiffusion of cathepsins at pH 8.0.

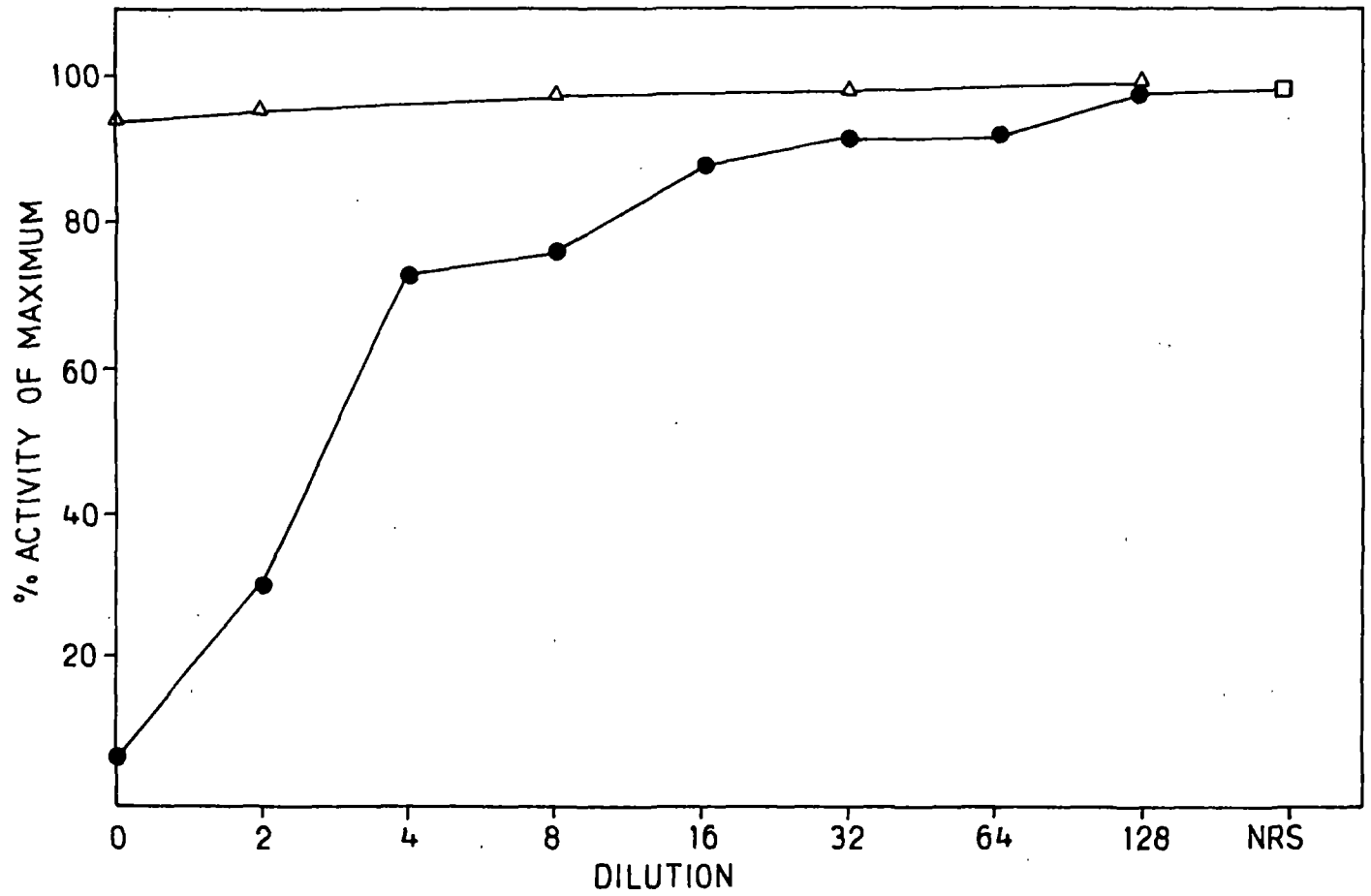
About 200 µl of antiserum was applied in the central well. The peripheral wells, starting from 1 clockwise contained buffalo kidney cathepsin B, bovine spleen cathepsin B, buffalo kidney cathepsin H and goat spleen cathepsin B. All the cathepsins were alkaline pH denatured prior to immunodiffusion.


1
2
3
4

distinct precipitin lines were formed, recognising cathepsin B under both the conditions (Fig. 34 & 35). The results thus showed that the epitope is insensitive to inhibitors and pH.

Binding of anti-buffalo kidney cathepsin B antiserum to cathepsin B resulted into inactivation of the latter (Coetzer **et al.**, 1991; Rowan **et al.**,1992). This inactivation process was highly specific because similar experiments with cathepsin H did not result into any loss of the enzymatic activity on its exposure to anti cathepsin B (Fig. 36). The inactivation of cathepsin B was reversible as revealed by dilution experiment described in the method section.

Fig. 36. Binding of anti-buffalo kidney cathepsin B antibody to cathepsin B and H.
Fixed amounts of cathepsins were incubated with different dilutions of anti-buffalo kidney antiserum raised in rabbits. Assay of residual activity were measured for cathepsin B (●——●) and cathepsin H (△——△). Normal rabbit serum(□)(NRS) was used for dilution of antiserum.


100
80
60
40
20
% ACTIVITY OF MAXIMUM
0
2
4
8
16
32
64
128
NRS
DILUTION

DISCUSSION

Although a number of methods have been previously described for the isolation and purification of cathepsin B from various sources there was always the felt need to devise methodology suitable for a particular source in terms of its simplicity, reproducibility and better yielding. The methodology developed in this study fulfills the above criteria while dealing with kidney as a source of cathepsin B.

Apart from other significant changes made in the purification procedure of cathepsin B (Ahmad **et al.,** 1989), we used CM Sephadex in our purification scheme as summarised in table II . Our choice of CM Sephadex over DEAE Sephadex was due to the better separation of cathepsins viz, cathepsin B and H with the former than the latter. Thus, this procedure could effectively be used for simultaneous purification of both cathepsin B and cathepsin H from the same source. About 3.57 mg of cathepsin B, purified over 168 fold, was obtained from 600 grams of buffalo kidney.

The purified enzyme was found to be homogeneous with respect to charge and mass as evident from the polyacrylamide gel electrophoretic studies, both under native as well as denaturing conditions. The single symmetrical protein peak of the enzyme obtained on gel filtration column was found to be fully superimposable with its activity peak suggesting that the enzyme was homogeneous with respect to size as well.

The molecular weight of the purified enzyme, both by analytical gel filtration and SDS-PAGE studies, came out to be very close (around 26 kDa) to what has been reported (23-29 kDa) for this enzyme from other sources (MacGregor **et al.**, 1978; Bajkowski **et al.**, 1983; Takahashi **et al.**, 1986; Agarwal **et al.**, 1987(b); Ahmad **et al.**, 1989). However, the absence of multiple protein bands in the PAGE and SDS-PAGE studies (Fig. 8 & 13), as suggested by Fazili and Qasim, (1986); Agarwal **et al.**, (1987b); Ahmad **et al.**, (1989), led us to believe that this enzyme lacked subunit structure and/or isozymes. This result may not be surprising since the earlier workers used liver and spleens as sources which shows histolytically much more complexity than the kidney tissues.

The isoionic pH for buffalo kidney cathepsin B was found to be 5.1, which falls well in the range of 4.8-5.3 as reported for this enzyme from different sources (Barrett **et al.**, 1981; Tanaka **et al.**, 1984).

The result on end group analysis of buffalo kidney cathepsin B are striking for they show alanine as the NH_2-terminal residue as against leucine for this enzyme from other tissues/species (Takio **et al.**, 1983; Takahashi **et al.**, 1984a, 1986; Ritonja **et al.**, 1985; Meloun **et al.**, 1988). This may either be attributed to simple species dependence or more significantly to the possible differential post translation processing of the enzyme in the kidney tissues (Katunuma **et al.**, 1983; Docherty **et al.**, 1984; Bachet **et al.**, 1986; Hasnain **et al.**, 1992). However,

the COOH-terminal amino acid residue was found to be threonine, in consistence with those reported for the enzyme from other sources (Meloun **et al.**, 1988; Takio **et al.**, 1983; Ritonja **et al.**, 1985; Ahmad **et al.**, 1990).

Increase in the number of sulfhydryl groups (from 0.6 to 1.6 mol/mol of protein) after exposure of the enzyme to denaturing conditions suggests the presence of an additional free cysteine residue besides the active site cys-29 which might be partially buried under native conditions (Otto, 1971; Takio **et al.**, 1987; Baudys **et al.**, 1991; Musil **et al.**, 1991; Sumiya **et al.**, 1992).

The total carbohydrate content of the buffalo enzyme was also found to be significantly lower (3.6%) than the reported values (about 7.6%) for the enzyme from other sources (Takahashi **et al.**, 1986; Ahmad **et al.**, 1989). This finding is in accordance with the subdued hydration noticed for buffalo kidney cathepsin B as compared to the data obtained for this enzyme from other sources (Fazili **et al.**, 1986; Ahmad **et al.**, 1989).

Amino acid composition of the buffalo kidney cathepsin B showed close similarities with its counterpart from other sources like bovine, porcine, human and rat tissues with exception to serine (Tab. VIII). The enzyme also contained significant amount of tryptophan residues. The absorption maxima and the emission maxima for the native enzyme protein was found to be at about 279 nm and 335 nm respectively. The extinction coefficient ($E^{1\%}_{1cm}$) of the kidney enzyme with the value of 16.78 was higher than its

counterpart from buffalo spleen (13.2, Ahmad **et al.,** 1989) but lower than that from human liver (20.0, Barrett **et al.,** 1981).

The striking differences in the degree of glycosylation as well as the number of serine residues of buffalo kidney cathepsin B as compared to the enzyme from other sources might be responsible for the reversed trend of binding of this enzyme in CM-Sephadex (Takahashi **et al.,** 1984a and figure 5 of this study).

Physicochemical parameters like temperature, pH, ionic strength and radiation exerted profound effect on activity and stability of the kidney enzyme. At temperature and pH levels above the physiological values, the enzyme was irreversibly inactivated. The involvement of several ionising groups, presumably, histidine imidazole groups (since the pH optima is 6.8), in the catalytic process might be responsible for this behaviour of the enzyme (Khouri **et al.,** 1991; Hasnain **et al.,** 1992; Sumner **et al.,** 1993). This experiment rules out the possible contamination of our enzyme preparation with cathepsin S like activity which show appreciable stability at alkaline pH and activity in the range of pH 4.0-5.0 (Mason **et al.,** 1986; Kirschke **et al.,** 1989; Wiederanders **et al.,** 1992 McDonald **et al.,** 1993).

The kidney enzyme was found to be sensitive against ionic strength. The enzyme showed appreciable activity in the salt concentration range of 12-25 mM which incidentally is the physiological ionic strength for most biological fluids. At higher salt concentration or buffer strength there was significant loss of activity. Hence the concentration of the assay buffer was re-

stricted to 20 mM. Since the loss of activity was reversible, long term storage of the enzyme was done in excess salt concentration (Khan **et al.,** 1987; Agarwal **et al.,** 1987(a).

The kidney cathepsin B was very sensitive to radiation also. At very low γ-radiation dose (2-20 Gy), the enzyme showed appreciable activity. At higher doses of the radiation (>30 Gy), however, the loss of activity was significantly higher. On comparison, it was found that the enzyme from buffalo kidney is more sensitive to γ-irradiation than its counterpart from buffalo or goat spleen (Ahmad **et al.,** 1988; Agarwal **et al.,** 1987b).

As evident from the result summarised in Table IX, The enzyme required the presence of reducing agents like cysteine base, DTT, 2-mercaptoethanol etc. for exhibiting its activity in the mild acidic range. This clearly indicates that the isolated kidney enzyme indeed belongs to the cysteine proteinase class of enzymes (Barrett **et al.,** 1981; Kirschke **et al.,** 1983; Evans **et al.,** 1983; Bajkowski **et al.,** 1983; Hasnain **et al.,** 1992; Sumner **et al.,** 1993). Among the various reducing agents used, DTT was found to be the most effective while cysteamine-HCl was the least effective. This result is in consistence with those reported for beef spleen and pork liver cathepsin B (Evans **et al.,** 1983). However, for most enzymatic assay, 2-mercaptoethanol (2mM) was used because of its higher half life as compared to others (Evans **et al.,** 1983; Agarwal **et al.,** 1987 (a).

As reported for cathepsin B from other sources, the activity of the buffalo kidney cathepsin B was suppressed with increas-

ing concentration of thiol group blocking compounds and heavy metal compounds like $HgCl_2$, $ZnSO_4$, p-chloromercuric benzoic acid; alkalyting agents like iodoacetic acid, iodoacetamide and peptidyl inhibitors like leupeptin, antipain, chymostatin and E-64 (Tab.X & XI). These results again suggest the involvement of cysteine and histidine groups in the expression of catalytic activities of the buffalo enzyme (Mac Gregor **et al.,** 1979; Lewis **et al.,** 1981; Polgar **et al.,** 1982; Bajkowski **et al.,** 1983 Hasnain **et al.,** 1992,1993; Sumner **et al.,** 1993). The indifference of the enzyme towards pepstatin ruled out the possibility of cathepsin D contamination in the enzyme preparation (Nishimura **et al.,** 1988). The remarkable inhibition of the enzyme by leupeptin and negligible hydrolysis of cathepsin H specific substrates like Arg-NA, Arg-MCA also suggested that the enzyme was essentially free from cathepsin H or leucine aminopeptidase contamination (Barrett **et al.,** 1981; Kirschke **et al.,** 1983; Takio **et al.,** 1983 Shaw **et al.,** 1983; Baudys **et al.,** 1991; Xin **et al.,** 1992; Bromme **et al.,** 1993).

The result on the effect of denaturants such as urea and guanidine-HCl on buffalo kidney cathepsin B suggested the kidney enzyme to be more sensitive to the latter as compared to the former. About 50% activity was lost at the urea concentration of about 1.0 M. These findings are in good agreement with what has been reported for the enzyme from buffalo spleen (Ahmad **et al.,** 1989). The reversibility of the lost activity was possible only when the enzyme was exposed to urea concentration less than 2.0M.

No activity could be recovered back when the enzyme was exposed to urea concentration of 3.0 M or above. This experiment also ruled out the possible contamination of the enzyme preparation with cathepsin L, which shows optimal catalytic efficiency even at 3.0 M urea and above (Barrett **et al.,** 1981).

The effect of Gdn-HCl on the activity of the purified buffalo kidney cathepsin B as summarised in figure 27, clearly shows that the enzyme lost its activity by 50% at the Gdn-HCl concentration as low as 0.01 M. The reversibility of the activity of the inactivated enzyme after dilution of the guanidium salt was possible when the enzyme was exposed below the Gdn-HCl of 0.5 M with full recovery at 0.1 M. No activity could be recovered back when the enzyme was exposed above 2.0 M Gdn-HCl.

Summing up the results obtained through the denaturant studies on the activity of cathepsin B it was observed the concentration of denaturants required to fully inactivate the enzyme was too low to fully denature it. As such , some minor "perturbations" at or around the active site of the enzyme , without effecting the tertiary structure might be accounted for this behaviour (Agarwal **et al.,** 1988; Khan **et al.,** 1992).

The antiserum raised in rabbits against the buffalo kidney cathepsin B was found to be specific for cathepsin B only and did not cross react with other cathepsins viz., cathepsins H and L from the same or different sources. The antiserum could recognise both the native as well as pH denatured buffalo cathepsin B. This, in contrast to earlier reports on cathepsin B from other

sources (Barrett, 1973; Mort **et al.,** 1980) shows that the buffalo enzyme does not undergo rapid denaturation following immunization.

The analysis of the best fit kinetic plots of cathepsin B reveal that the kidney enzyme has great catalytic potential against synthetic as well as protein substrates. The increase K_m of the kidney enzyme as against its counterpart from spleen and liver does suggest a species or tissue specificity of this enzyme (Barrett **et al.,** 1981; Towatari **et al.,** 1983; Takahashi **et al.,** 1984a,b; 1986; Kominami **et al.,** 1985; Bando **et al.,** 1986; Bechet **et al.,** 1986; Chan **et al.,** 1986; Hara **et al.,** 1988; Nishimura **et al.,** 1988; Pagano **et al.,** 1988; Hasnain **et al.,** 1992)

Cathepsin B has long been demonstrated to be active against aldolase, thereby inactivating the latter (Towatari **et al.,**.1979; Bond **et al.,** 1980). Our studies on buffalo kidney cathepsin B also supports this by giving positive test. However, the enzyme from buffalo kidney showed a reduced affinity against aldolase as substrate as against its counterpart from other sources. Only about 25% inactivation of aldolase was observed as against 55-60% inactivation observed with porcine lungs cathepsin B under our assay condition. The reported value for aldolase inactivation from other sources like porcine liver and bovine enzymes was about 75-80% (Takahashi **et al.,** 1986).

In the light of our findings that have been discussed above, it can be concluded that a simple methodology for simultaneous purification of cathepsin B and H from buffalo kidney has

been developed.

Although the enzyme purified by us showed close similarities with its counterpart from other sources with respect to molecular weight, catalytic nature and response to reducing agents and inhibitors, yet, it differs from others with respect to NH_2-terminal group, carbohydrate contents, serine contents, catalytic efficiency against several natural protein substrates like BSA, casein, haemoglobin and aldolase as well as synthetic peptide substrates like Z-Phe-Arg-MCA, Z-Arg-Arg-MCA, BAPNA, BANA. An striking feature of the enzyme purified from the buffalo kidney is that it exists in a single polypeptide chain form and as such does not have isozyme forms. All these finding taken together therefore suggest a strong species and/or tissue dependence of cathepsin B from mammalian sources (Barrett **et al.**, 1981; Towatari **et al.**, 1983; Takahashi **et al.**, 1986; Bando **et al.**, 1986; Bechet **et al.**, 1986; Kominami **et al.**, 1986; Pagano **et al.**, 1988; Hara **et al.**, 1988; Hasnain **et al.**, 1992 Mach **et al.**, 1992).

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