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), cytochrome 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 aminoethyl (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. 81P-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'-tetramethylethylenediamine, 2-mercaptoethanol, coomassie brilliant blue R-250, glycine, 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-naphthylamine, p-nitroaniline, DTNB, FCA, IFA, antipain, chymostatin, E-64, leupeptin, pepstatin A, cysteine base, cysteamine-HCl, dithiothreitol, glutathione, thioglycerol, iodoacetic acid, p-mercuric benzoic acid, guanidine hydrochloride, 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-naphthylethylene diamine-HCl and standard buffer tablets of different pHs were purchased from BDH, England. Agarose, Ammonium sulfamate, EDTA, histidine, papain, methyl cellulose, 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 spectrofluorophotometer, 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 content 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 frothing 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<sup>1%</sup>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 5 cm 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 (K₃(FeCN)₆) solution (2 mg/ml). The elution volume of the former gave the void volume (V₀), while that of the latter gave the total volume (Vₜ) 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 9.0 cm 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:

$$R_m = \frac{\text{Distance traversed by the protein band (cm)}}{\text{Distance traversed by the marker dye band (cm)}}$$
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 chromatographed on a Sephadex G-75 column (2.6x90 cm) pre-equilibrated with 50 mM sodium acetate buffer, pH 5.0 containing 1mM 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 chromatography 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 spectrofluorometrically. 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 histidine buffer, pH 6.3, containing 1 mM EDTA and 1.4 mM 2-mercaptoethanol. 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 (H₂O₂) 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 8.6x10^4 /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,
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:

\[ C = \frac{AxD}{E} \]

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) H₂SO₄ 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 NaNO₂ (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: 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-naphthylamine thus released was calculated from the standard curve prepared in similar manner with varying concentration of 2-naphthylamine.

(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 spectrofluorimeter 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) Aminomethylcoumarin 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 wavelengths 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

0.25 ml of enzyme solution was incubated with equal volume
of activator buffer (20 mM sodium phosphate buffer, pH 6.5, con-
taining 10 mM cysteine base and 1.0 mM each of EDTA and pepsta-
tin) 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 oc-
curred 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 hydridantin 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 methylcellulose) 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,

$$\frac{1}{v} = \frac{K_m}{V_{max}} \left\{ \frac{1}{[s]} \right\} + \frac{1}{V_{max}}$$

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
\( E_{1\%}^{1\text{cm}} \) 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 dialy-
sed 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 (\( t_0 \)), were re-
corded with the help of a stop watch having a least count of 0.1
sec. The intrinsic viscosity \( [\eta] \), of the protein solution was
computed by the method of Tanford (1955) using the following expression:

\[
\eta = \lim_{C \to 0} \left( \eta - \eta_0 \right) / \eta_0 \cdot C \\
= \lim_{C \to 0} \left[ \left( t - t_0 \right) / t_0 \cdot C \right] + \left( 1 - \bar{v}_2 \rho_0 / \rho_0 \right)
\]

where, \( \eta_0 \) and \( \eta \) were the viscosities in poise of the solvent (buffer) and the protein solution respectively, \( C \) was the protein concentration in g/ml, \( \rho_0 \) 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 \( \mu \)g of the purified (and extensively dialysed against distilled water) protein \( \text{conjugated with complete Freund's adjuvant} \) was injected intramuscularly followed by two booster doses (in incomplete Freund's adjuvant) 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 \( \mu \)g) or cathepsin H (0.5 \( \mu \)g)
were incubated at 37°C for 1 hr. with 0.1 ml of antiserum pro-
duced 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.