MATERIALS

The following tubers were procured from local sources: Alocasia (Alocasia macrorhiza), arrow root (Maranta arundinacea), coleus (Coleus perviformis), colocasia (Colocasia antiquorum), potato (Solanum tuberosum), sweet potato (Ipomoea batatas), tapioca (Manihot utilissima) and yam (Amorphophallus campanulatus).

Bovine trypsin (salt free, thrice crystallized, E.C. 3.4.21.4) and bovine α-chymotrypsin (salt free, thrice crystallized, E.C. 3.4.21.1) were the products of Worthington Biochemical Corporation, U.S.A. Pronase (Type VI protease), peroxidase, cytochrome c, myoglobin, α-N-benzoyl L-arginine ethyl ester (BAEE), α-N-benzoyl DL-arginine p-nitro anilide (BAPNA), 2,4,6-trinitrobenzene sulphonate (TNBS), O-methylisourea (OMI), α-N-acetyl L-tyrosine ethyl ester (ATEE), acrylamide, N,N'-methylene (bis) acrylamide, 2-mercaptoethanol, ammonium persulphate, N,N,N',N''-tetramethyl erylene diamine (TEMED), acetone powders of dog, rat, cat, rabbit, porcine, equine and ovine pancreases, bovine serum albumin and Dalton mark VI were purchased from Sigma Chemical Company, St. Louis, U.S.A. Porcine pepsin (thrice crystallized) and vitamin free casein were obtained from Calbiochem, U.S.A.

DEAE-cellulose (Cellex-D), sodium dodecyl sulphate (SDS), Coomassie brilliant blue R-250 and Affigel-10 were the
products of Bio-Rad Laboratories, U.S.A. Sephadex G-100, Sephadex G-15, Blue dextran-2000 and Sephacryl 3-200 were from Pharmacia Fine Chemicals, Sweden. Ninhydrin was obtained from Pierce Chemicals, U.S.A. 1,2-Cyclohexanedione(CHD) was purchased from Aldrich Chemicals, U.S.A. Riboflavin was from E. Merck, AG Dermstadt, Germany. Subtilisin BPN' was purchased from Nagase Company, Osaka, Japan. Bovine pancreas was collected from a local abattoir. Human pancreas was obtained during autopsy at Kasturba Medical College Hospital, Manipal. All other chemicals used were of analytical grade commercial samples.

**GENERAL METHODS**

Preparation of acetone powder of bovine and human pancreas:

Acetone powder preparation of bovine and human pancreas, which served as the sources of the proenzymes of the pancreatic enzymes were prepared as follows at 4°C.

Pancreata obtained were homogenized with 3 volumes (w/v) of 0.02 M sodium phosphate buffer pH 7.6 in a Waring blender. To the homogenate 5 volumes of precooled (-5°C) acetone were added dropwise and the mixture was stirred for 20 minutes. The residue was collected by suction filtration, washed thoroughly with cold acetone and finally with peroxide free ethyl ether. The preparations, designated as acetone powder, were stored at 4°C under desiccation.
Activation of acetone powder of bovine and human pancreas:

The acetone powder preparations of bovine pancreas and human pancreas were activated by treating 500 mg of the powder in 10 ml of 0.02 M borate buffer pH 7.6 containing 0.05 M CaCl₂ with one mg (protein) of bovine enterokinase preparation for one hour at 37°C. The suspension was centrifuged at 2500 g for 10 minutes and the clear supernatant was used as the source of pancreatic proteases.

Partial purification of bovine enterokinase:

Enterokinase was purified partially by the method of Liepnicks and Light operating at 4°C(234). Mucosal cells were collected by scraping from duodenum and jejunum and homogenised with 50 ml of 0.1 M Tris-HCl buffer pH 8.0 containing 4 gm of sodium deoxycholate. The suspension was centrifuged at 10 000 g for 20 minutes. The pH of the supernatant was adjusted to 6.0 with 1 M acetic acid. The precipitate formed was removed by centrifugation at 10 000 g for 20 minutes. The supernatant was subjected to ammonium sulphate fractionation after adjusting the pH to 8.0 with 1 N NaOH. The precipitate formed in the range of 40-70% ammonium sulphate saturation was collected, suspended in 10 ml of 5 mM Tris-acetate buffer pH 6.0 containing 20 mM NaCl, and dialyzed overnight against 10 volumes of the same buffer. The dialyzed solution was centrifuged at 10 000 g for 10 minutes and the clear supernatant was used as the source of enterokinase.
Measurement of activities of neutral proteases and their inhibitors:

The proteolytic activity of neutral protease and inhibition of proteolytic activity were determined by the caseinolytic method of Kunitz with the following modifications (80). The assay system consisted of 1 ml of the buffered casein substrate solution, 0.2 ml of 0.2 M phosphate buffer pH 7.6 and water in a total volume of 1.8 ml. The reaction was started by the addition of 0.2 ml of suitably diluted enzyme solution. After 10 minutes incubation at 37°C, the reaction was stopped by adding 3 ml of 5% (w/v) trichloroacetic acid. This was used as the enzyme control. To determine the inhibition of proteases, different aliquots of the inhibitor in a volume of 0.6 ml were included in the incubation mixtures and the assay was performed in a similar way like enzyme control. Enzyme and inhibitor blanks were run simultaneously in which 0.2 ml of suitably diluted enzyme was added after the addition of trichloroacetic acid.

After standing for 45 minutes the reaction mixtures were centrifuged at 2500 g for 15 minutes. The clear supernatant was analyzed for trichloroacetic acid soluble fragments by the method of Lowry et al (235) as follows.

To one ml of clear supernatant one ml of water was added followed by four ml of alkaline copper reagent. After 10 minutes, 0.4 ml of 1 N Folin-Ciocalteu reagent was added.
The blue color developed was measured after 30 minutes at 540 nm.

**Enzyme solutions:**

Stock solutions of trypsin and chymotrypsin were prepared by dissolving 10 mg of the respective enzyme in one ml of $10^{-3}$ M hydrochloric acid. The stock enzyme solutions were stored at 4°C. The stock enzyme solutions were diluted with distilled water just before use. The amount of enzymes used in the caseinolytic method were 10 μg of trypsin, 12.5 μg of α-chymotrypsin, 12.5 μg of elastase and 20 μg of subtilisin BPN'.

**Preparation of buffered casein substrate solution:**

Two grams of casein was dissolved in 40 ml of 0.1 N sodium hydroxide with the help of heat at 60°C. This solution was cooled and 10 ml of 0.2 N hydrochloric acid was added dropwise with constant mixing to get a pH of 7.6. The volume was made upto 100 ml with 0.2 M phosphate buffer, pH 7.6. The buffered casein was stored at 4°C for a maximum of 4 days.

The increase in the concentration of the trichloroacetic acid soluble fragments obtained with caseinolytic method was found to be linear with increasing concentration of either trypsin or chymotrypsin upto an optical density value of 0.60. The linearity curve of trypsin and α-chymotrypsin are shown.
FIGURE 1 - EFFECT OF ENZYME CONCENTRATION ON HYDROLYSIS OF CASEIN

- Trypsin
- Chymotrypsin
Enzyme concentrations used in the assay system were adjusted in such a way that the optical density values obtained were around 0.60. Inhibitor concentrations were used in such a way to get 25-50% inhibition of either trypsin or chymotrypsin.

One unit of enzyme is defined as the amount of enzyme that will cause an increase of one mg of Lowry sensitive material in the trichloroacetic acid soluble fraction under the assay condition. One unit of inhibitor is the amount of inhibitor which will depress the proteolytic activity of the enzyme by one unit. For calculations, the values were determined based on a reference curve using bovine serum albumin as standard in Lowry procedure.

**Estimation of proteins:**

Protein was estimated by the method of Lowry et al using bovine serum albumin as standard (235). The samples containing (40-400 μg of protein) in a total volume of 2.0 ml were treated with 4.0 ml of freshly prepared alkaline copper reagent followed by 0.4 ml of Folin-Ciocalteu reagent after 10 minutes. The contents were mixed thoroughly. The blue color developed was read at 540 nm after 30 minutes.

**Measurement of albuminolytic activity of trypsin and α-chymotrypsin:**

The albuminolytic activities of trypsin and chymotrypsin were estimated by using bovine serum albumin as substrate.
The assay system consisted of 1.0 ml of buffered albumin solution, 0.2 ml of 0.2 M phosphate buffer pH 7.6 and water in a total volume of 1.8 ml. The reaction was started by the addition of 0.2 ml of suitably diluted enzyme solution. The remaining procedure for measurement of albuminolytic activity and inhibitory activity was same as the procedure described for caseinolytic method.

The increase in the concentration of TCA soluble fragments with this method were linear upto an optical density of 0.50 with trypsin and 0.30 with chymotrypsin. The linearity curves for trypsin and chymotrypsin are shown in Fig. 2 (Page 65). The amount of enzymes used in the albuminolytic method were 30 μg of trypsin and 72.5 μg of chymotrypsin.

Preparation of albumin substrate:

Two g of albumin was dissolved in 100 ml of 0.1 M phosphate buffer pH 7.6 and stored at 4°C for a maximum of 4 days.

Measurement of amidolytic activity of trypsin:

The amidolytic activity of trypsin was estimated as described by Erlanger et al (236) using α-N-benzoyl DL-arginyl p-nitroanilide as substrate. The assay system consisted of 5 μmoles of substrate (prepared as described below), 100 μmoles of Tris-HCl buffer, pH 8.0 and 1 μmole of calcium chloride in a total volume of 2.8 ml. The reaction was started by the addition of 0.2 ml of diluted
FIGURE 2 - EFFECT OF ENZYME CONCENTRATION ON HYDROLYSIS OF ALBUMIN

- • Trypsin
- ○ Chymotrypsin
trypsin (10 μg of trypsin). After 30 minutes of incubation at 37°C, the enzyme reaction was arrested by the addition of 1 ml of 30% (v/v) acetic acid. The p-nitroaniline liberated was measured at 410 nm. Enzyme and inhibitor blanks were performed simultaneously in which enzyme was added after the addition of acetic acid. The linearity curve with trypsin is shown in Fig. 3A (Page 67).

**Preparation of substrate:**

Stock substrate solution: 0.1 M solution of α-N-benzoyl DL-arginyl p-nitroanilide was prepared by dissolving 435 mg of solid in 10 ml of dimethyl sulfoxide and stored at 4°C.

Substrate for use: 1.0 ml of stock substrate solution was diluted to 20 ml with 0.1 M Tris-HCl buffer pH 8.0.

**Measurement of esterolytic activity of chymotrypsin:**

This was done according to the method of Prabhu and Pattabiraman (237) using α-N-acetyl L-tyrosine ethyl ester. The assay system consisted of 10 μmoles of substrate solution (prepared as described below), 100 μmoles of phosphate buffer pH 7.6 and inhibitor solution or water in a total volume of 1.8 ml. The reaction was started by adding 0.2 ml of enzyme (1.25 μg of chymotrypsin). After 10 minutes of incubation at 37°C, the reaction was stopped by shaking the solutions with 5 ml of ethyl acetate. The acetyl tyrosine
FIGURE 3 - A. EFFECT OF TRYPSIN CONCENTRATION ON HYDROLYSIS OF BAPNA
B. EFFECT OF CHYMOTRYPSIN CONCENTRATION ON HYDROLYSIS OF ATEE
formed by enzyme hydrolysis and which remained in the aqueous layer was estimated by the method of Lowry and coworkers (235). Enzyme and inhibitor blanks were run in similar way except that the enzyme was added after the addition of ethyl acetate. The optical densities of acetyl tyrosine measured by Lowry's method were linear up to a value of 0.60 with increasing concentration of α-chymotrypsin. The linearity curve with α-chymotrypsin is shown in Fig. 3B (Page 67).

**Preparation of substrate:**

This was made by dissolving 126 mg of α-N-acetyl L-tyrosine ethyl ester in 50 ml of 0.1 M phosphate buffer, pH 7.6 at 60°C. The substrate solution was stored at 4°C when not in use for a maximum of 4 days.

**Esterase activity of bovine enterokinase:**

The esterolytic activity of enterokinase was estimated as described by Bhat et al (238) using α-N-benzoyl L-arginine ethyl ester (BAEE) as substrate. The assay system consisted of 2 μmoles of substrate, 20 μmoles of Tris-HCl buffer pH 8.0, 1 μmole of calcium chloride, 30 μl of enzyme (250 μg protein) and water in a total volume of 1.0 ml. After 1 hour incubation at 37°C the reaction was stopped by the addition of 0.2 ml of 10% (w/v) trichloroacetic acid. After 30 minutes of standing, the solution was centrifuged at 2 000 g for 10 minutes. The supernatant (0.5 ml) was applied on a column
of Sephadex G-15 (bed volume 5 ml, 0.8 cm x 10 cm) and eluted with water at flow rate of 20-25 ml/hr. The first 7 ml was discarded. The next 6 ml was collected and 4 ml aliquot of this was used for the estimation of benzoyl arginine. For inhibitory assay, inhibitor was included in the assay system in suitable aliquots. Enzyme and inhibitor blanks were run simultaneously in similar way except that the enzyme was added after the addition of trichloroacetic acid.

Estimation of benzoyl arginine:

\(\alpha\)-N-benzoyl arginine formed during hydrolysis of \(\alpha\)-N-benzoyl arginine ethyl ester by enterokinase was estimated by the Sakaguchi reaction (239). To 4 ml of ice-cold aqueous solution containing the benzoyl arginine, 1 ml of cold \(\alpha\)-naphthol urea solution (0.2% naphthol in 95% alcohol diluted with 4 volumes of 10% urea in water prior to use) was added with mixing. After 2 minutes, 0.4 ml of ice-cold fresh hypobromite solution (7.5 ml of saturated bromine water mixed with 2.5 ml of 20% sodium hydroxide) was added from a pipette with a fine bore, with mixing. After 20 minutes in the ice bath, the color was measured at 525 nm. The optical densities of \(\alpha\)-N-benzoyl arginine measured by this method were linear up to a concentration of 150 nmoles.
Estimation of carbohydrate:

Total carbohydrates in the inhibitors were estimated according to the method of Dubois et al (240) using galactose as standard. The standard sugar solution or the inhibitor solution in a volume of 2.0 ml was taken and 0.05 ml of 80% phenol was added and mixed thoroughly. Then 5 ml of concentrated sulphuric acid was added and the colour was measured at 480 nm after 30 minutes. A water blank was run simultaneously.

Active site titrations of pure trypsin and chymotrypsin:

The active site titrations of pure trypsin and chymotrypsin were done as described by Kezdy and Kaiser (241) using p-nitrophenyl acetate as the substrate. The bovine trypsin was found to be 65% active whereas 55% activity was obtained in case of α-chymotrypsin.

Electrophoresis:

Cellulose acetate membrane electrophoresis of the purified inhibitor was performed at pH 8.6 (0.05 M barbitone buffer) and at pH 5.0 (0.5 M acetate buffer) for 75 minutes at 200 V. The strips were stained with 0.1% Coomassie brilliant blue R-250 in 7% acetic acid.

Polyacrylamide gel electrophoresis was performed according to the method of Davis et al (242) at pH 8.3 using 7% polyacrylamide. The electrophoresis was performed...
for 60 minutes at 3 mA current per tube. Coomassie brilliant blue R-250 (0.1% in methanol-acetic acid-water 5:1:5 v/v) was used to stain the protein.

Polyacrylamide gel electrophoresis of the protein samples was performed in presence of sodium dodecyl sulphate as described by Weber et al (243) in 10% polyacrylamide. Sodium phosphate buffer (0.1 M), pH 7.2 containing 0.1% SDS (w/v) was used both for preparation of gel and in electrode chamber. Suitable amounts of sample were incubated with 0.01 M phosphate buffer pH 7.2 containing 1% SDS, 1% mercaptoethanol and 36% urea for 2 hours at 37°C. The electrophoresis was performed for 5 hours at 7 mA current for each tube. Staining was done as described above.

Molecular weight determination by gel chromatography:

The molecular weights of the purified inhibitors were determined by gel chromatography on Sephadex G-100 column (0.9 x 56.5 cm, bed volume 36 ml). The column was equilibrated with 0.02 M phosphate buffer pH 7.6 containing 0.1 M NaCl. The inhibitor samples in 1 ml of equilibration buffer were applied to the column and the elution volume (Ve) was determined. The void volume (Vo) was determined using Blue dextran-2 000. The Ve/Vo values of the inhibitors were compared with those of different marker proteins which were also processed in a similar manner through the column. In all the cases one ml fractions were collected at a flow rate of 8 ml/hr and the protein contents of the
fractions were estimated by the method of Lowry et al (235). Cytochrome c (molecular weight 13 000), myoglobin (17 800), trypsin (24 000), peroxidase (40 000) and bovine serum albumin (66 000) were used as marker proteins.

**Molecular weight determination by SDS-polyacrylamide gel electrophoresis:**

Molecular weights of the purified inhibitors were also determined by polyacrylamide gel electrophoresis in presence of SDS according to the method of Weber et al (243) as described above. The inhibitor samples were treated with 1% SDS, 1% mercaptoethanol and 36% urea in 0.01 M phosphate buffer pH 7.2 at 37°C for 2 hours and subjected to electrophoresis in presence of bromophenol blue. The ratios between the mobility of protein bands and bromophenol blue were calculated. To determine the molecular weights of the inhibitors the mobility ratios were compared with those of different marker proteins which were processed similarly. Dalton mark VI was used as the source of six different marker proteins, lysozyme (hen's egg white, molecular weight 14 300), β-lactoglobulin subunit (18 400), trypsinogen (24 000), porcine pepsin (34 700), ovalbumin (45 000) and bovine serum albumin (66 000). Other methods employed in this study are described under respective chapters.