CHAPTER III

EXPERIMENTAL METHODOLOGY

The research project was divided into two major sections :

1. Studies of chitosans as nasal absorption enhancer of sCT using in vivo nasal absoption technique.

1.1 Studies to determine the optimum enhancing conditions for each of the two chitosans,i.e. free base (CS J) and glutamate salt(CS G).

1.2 Comparison of the efficacy between chitosans and cyclodextrin derivatives in improving nasal absorption of sCT.

2. Inhibitory effect of chitosans on the activity of two nasal proteolytic enzymes, leucine aminopeptidase and trypsin, using in vitro technique.

Animal Model :

Male Sprague-Dawley rats weighing 250-300 g were obtained from National Laboratory Animal Centre, Mahidol University, Nakornpatom, Thailand.

Materials :

Chitosan J (CS J) = Chitosan, Kyowa Technos Co., Ltd., Japan.

Chitosan G (CS G) = Chitosan Glutamate Salt (Seacure G 210+), Lot No.206-409-03, Pronova Biopolymer, Drammen, Norway.

Thyrocalcitonin Salmon (Synthetic) , Lot No. 84H4951 and Lot No. 84449512, Sigma Chemicals Co., St Louis, USA.

Calcitonin (Salmon) RIA kit, RIK-600, Lot No. 970311, Peninsula Laboratories Inc., Belmont, California, USA.

Calcium Kit, Lot No. 628756 and 958456, Clinag Co. Ltd, Bangplad, Bangkok , Thailand.

Hydroxypropyl -β-Cyclodextrin, Lot No. 231026R, Aldrich Chemical Co. Inc., Milwaukee, USA.

Heptakis (2,6-Di-0-Methyl)-β-Cyclodextrin, Lot No. 56H0347, Sigma Chemicals Co., St Louis, USA.

Thiopental, Lot No.1300495, Research Institute of Antibiotics and Biotransformation, Czech Republic.

Heparin, Lot No. 01976A, Leo Pharmaceutical Products, Ballerup, Denmark.

Sodium Chloride AR grade, Lot No.4F027304F, Farmitalia Carlo Erba, Milano, Italy.

Sodium Hydroxide AR grade, Lot No. 191293, Eka, Sweden.

Ethanol Absolute GR grade, Lot No.321K19712083, E. Merck, Darmstadt, Germany.

Disodium Hydrogenphosphate AR grade, Lot No. F997086, E. Merck, Darmstadt, Germany.

Sodium Phosphate Monobasic AR grade, Lot No. G37948D5560A, Farmitalia Carlo Erba, Milano, Italy.

Phosphoric Acid 85% GR grade, Lot No. 931K42677273, E. Merck, Darmstadt, Germany.

Trypsin (From bovine pancreas), Lot No. 63H02802, Sigma Chemicals Co.,St Louis, USA.

N-2-Benzoyl-DL-Arginine- p-Nitroanilide Hydrochloride (BAPA HCl) Lot No. 25H1133 and Lot No. 16H0310, Sigma Chemicals Co., St Louis, USA.

p-Nitroaniline, Lot No. 85K3406, Sigma Chemicals Co., St Louis, USA.

Trichloroacetic Acid GR grade, Lot No. 637K2887707, E. Merck, Darmstadt, Germany.

Aprotinin (From bovine lung), Lot No.75H7186, Sigma Chemicals Co., St Louis, USA.

Leucine Aminopeptidase (Type VI-S : From porcine kidney microsomes), Lot No. 61H7045, Sigma Chemicals Co., St Louis, USA.

L-Leucine-β-Napthylamide Hydrochloride, Lot No. 025H6220, Sigma Chemicals Co., St Louis, USA.

N-1-Naphthylethylene-diamine DiHCl, Lot No. 105H6160, Sigma Chemicals Co., St Louis, USA.

Sodium Nitrite, Lot No.065H6131, Sigma Chemicals Co., St Louis, USA.

Ammonium Sulfamate, Lot No. 105H6159, Sigma Chemicals Co., St Louis, USA.

Alcoholic Dye Base, Lot No. 035h6211, Sigma Chemicals Co., St Louis, USA.

Bestatin, Lot No. 103H58601, and 103H58603 Sigma Chemicals Co., St Louis, USA.

Equipment :

UV Spectrophotometer, Model 7800, Jasco Corporation, Tokyo, Japan.

pH Meter, Model 420A, Orion Reasearch Operation, Boston, Massachusette, USA.

Analytical Balance, Sartorious 1615 MP, Range 300 gm/1 mg, Gottingen, Germany.

Thermostatted Circulating Water Bath, Heto InterMed, Heto Birkerod, Denmark.

Vortex Mixer, Model G-560E, Scientific Industries. Inc., New York, USA.

Ultrasonic Bath, Model 3210, Branson Ultrasonic Corporation, Danbury, Connecticut, USA.

Osmometer, Osmomat 030-D, Gonotec, Berlin, Germany.

Refrigerated Centrifuge, Model Sigma - 302 K, Order No. 10306, Laboratory Centrifuges, Federal, Germany.

Gammatec II, Model Gamma 600B Gamma counter, The Nucleus Inc.,Oak Ridge, USA.

Laminar Air Flow Hood, Model Airone 1000-GS, Serial No. AG015g, Safelab Systems Ltd, Nailsed, Great Britain.

Part I Studies of chitosans as nasal absorption enhancer of salmon calcitonin using in vivo nasal absorption technique

1.1 Studies to determine the optimum enhancing conditions for each of the two chitosans, i.e. free base (CS J) and glutamate salt (CS G).

1.2 Comparison of the efficacy between chitosans and cyclodextrin derivatives to improve nasal absorption of sCT.

In vivo nasal absorption technique was used to evaluate the effectiveness of different nasal formulations. This technique was first developed by Hirai et al. (1981) and has been successfully used by other researchers such as Hussain et al. (1985). It may be useful in screening of a drug candidate for nasal absorption and to confirm that the drug is actually absorbed across the nasal mucosa into systemic circulation. Furthermore, the technique also allows for the quantitation of the extent of nasal absorption (absolute bioavailability) as compared to the control (intravenous administration). The nasal absorption of sCT was evaluated by its hypocalcemic effects and measurements of its plasma level using in vivo nasal absorption technique. The following is a detailed description of the in vivo absorption procedure which is a slight modification of Hirai's method (Hirai et al., 1981).

Male Sprague-Dawley rats weighing 250-300 g were fasted for 16-18 hr prior to the experiments but allowed free access to water. They were anesthetized by intraperitoneal injection of sodium pentobarbital at a dose of 50 mg/kg, with additional doses given intraperitoneally as necessary. For surgery procedure, an incision was made at the neck of the

rats to expose the trachea. A polyethylene tube was inserted about 1.5 cm deep into the trachea toward the lungs to maintain respiration during the experiment. The esophagus was also cannulated with another similar polyethylene tube, which was closed at the end with an adhesive agent (Elephant Glue®) and inserted toward the posterior nasal cavity. Such manipulation was necessary to prevent the rat from swallowing the drug into the esophagus through nasopharynx. The nasopalatine was sealed with an adhesive agent to prevent drainage of the drug solution from the nasal cavity into the mouth. Respective diagrams of the surgical procedure of the in vivo nasal absorption experiment are illustrated in Figure 4 .

Nasal Administration of sCT

Drug solution was administered into the rat nasal cavity at a volume of 80-100 µl depending on the dose, through the nostril via a microsyringe (Hamilton; Reno, Nevada) which was attached to a blunt needle. The microsyringe was inserted into the right nasal cavity at a position about 4-5 mm toward the nostril and the drug solution was administered intranasally within 30 sec. The dose of sCT solution in all case for nasal administration was 10 IU/Kg body weight. Therefore, to achieve the dose and administration volume as required, the concentration of sCT in the final preparation was calculated to be 0.3 IU/ml (equivalent to 1.75 µM). Blood samples (0.4 ml each) were collected periodically from the jugular vein for 240 min (at 0, 10, 20, 30, 40, 60, 90, 120, 180 and 240 min) following administration. Following each blood withdrawal, the same volume of sterile normal saline was put back into the circulation to maintain the total blood volume and thus viability of the rats. The cannulation of the jugular

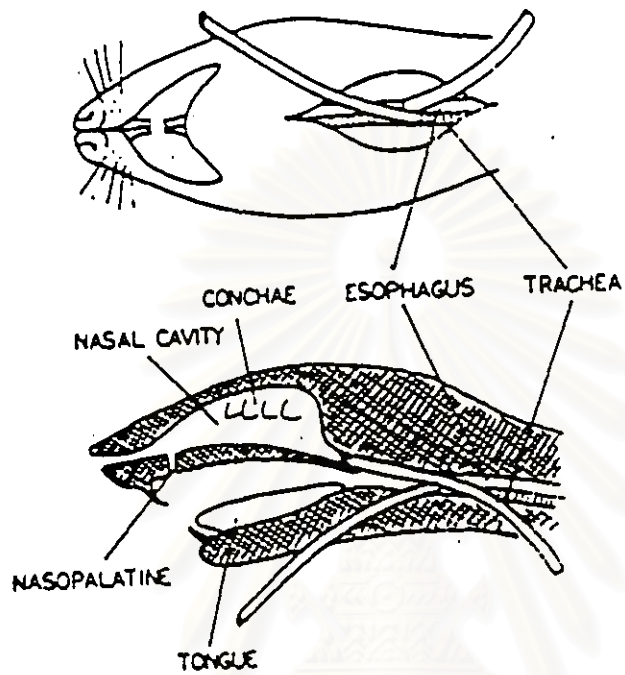

Figure 4 Diagrams of the surgical procedure of the in vivo nasal absorption experiment

vein was applied from the method of Harms and Ojeda (1974) using a catheter No. 20G (Jelco™ , Italy), filled with heparinized physiological saline (20 IU/ml) for blood sampling, i.v. administration of sCT and physiological saline.

Intravenous Administration of sCT

In order to determine the absolute bioavailability of nasally absorbed of sCT, intravenous administration was necessary. The same compound was administered intravenously via the jugular vein as a control to compare the areas under the plasma concentration-time curves between the two routes of administration. The iv. pharmacokinetic of sCT in rats was determined by injecting a bolus dose of 0.15 IU/Kg sCT in 0.15 M phosphate buffer (pH 4.0) into the jugular vein. To ensure complete dosing the injection cannula was flushed afterwards with 200 µl physiological saline. Blood samples of approximately 0.4 ml were withdrawn at 0, 5, 10, 15, 30, 60, 120 and 180 min after application and treated as described above.

At each specified interval, blood was collected into a heparinized microcentrifuge tube (Eppendorf® Safe-lock tube, Germany) and was centrifuged at 3,000 x g for 15 min using refrigerated centrifuge. Plasma samples were separated and kept frozen at -20° C for analysis of both plasma calcium level and immunoreactive sCT. The absorption of sCT through nasal mucosa was estimated indirectly from the reduction in plasma calcium and directly by measuring the plasma sCT concentration.

Preparation of Sample Solutions

The basic formulation of 3.5 μM sCT (6 IU/100 μl) was prepared by separately dissolving the in four pH's of 0.15 M isotonic phosphate buffer (IPB), i.e. pH 3.0, 4.0, 5.0 and 6.0. The stock solutions of two chitosans (CS J and CS G) were prepared at 2 % w/v concentration by dissolving each one in 1% v/v acetic acid solution and allowing them to swell overnight. The pH was subsequently adjusted to the same as the drug solution (pH 3.0, 4.0, 5.0 or 6.0) by dropwise addition of either 1 N hydrochloric acid or 1 N sodium hydroxide solution. The tonicity was then adjusted by stepwise addition of sodium chloride to obtain an isotonic solution which was checked by osmoter (290-310 mosmole/Kg). Chitosans were incorporated in the basic formulation before use. The sample solution containing 1 % w/v chitosan in 1.75 μM sCT was finally prepared by mixing the same pH solutions of 3.5 μM sCT (dose = 10 IU/Kg) and 2 % w/v chitosan in an equal portion. The concentration of sCT (1.75 μM) was fixed throughout the entire experiments whereas the concentration of CS J and CS G was also prepared at 0.25, 0.50, 0.75 and 1.25 % w/v. Similar method of preparation was applied by mixing chitosan stock solutions (0.5, 1, 1.25 and 2.50 % w/v) with 3.5 μM sCT solution (dose = 10 IU/Kg) at a 1:1 ratio. Table 4 lists the sample solutions which were prepared for investigation.

The effect of pH on the enhancing activity of both chitosans was first investigated by varying the pH of the sample solutions from 3.0 to 6.0 (concentration of chitosan fixed at 1% w/v). Furthermore, the baseline groups were carried out by nasal administration of only the phosphate

Table 4 Sample solutions prepared for experiment of Part I

Conditions	Dose of sCT (IU/Kg)	Enhancers	Conc. of enhancer (% w/v)	pH of sample solution in 0.15 M IPB				
				3.0	4.0	5.0	6.0	7.4
1. Baseline group	-	-	-	/	/	/	/	/
2. Control group	10	-	-	/	/	/	/	/
3. $sCT_{treated}$ group (sCT with enhancers)	10	CS J , CS G	1	/	/	/	/	-
		CS J , CS G	0.25 0.50 0.75 1.25	Optimum pH				
		DM-B-CD , HP-B-CD	5	-	-	-	-	/

IPB = Isotonic Phosphate Buffer

buffer at various pH (no sCT and no enhancers) in order to determine the baseline of calcium level for each pH. After determining the pH of optimal enhancing activity for each chitosan, the effect of chitosan concentration was subsequently studied at 0.25 % to 1.25 % w/v. The sample solution also contained sCT at the final conc. of 1.75 μM and was administered at the same dose. The same in vivo technique and analytical method were followed in order to determine the concentration which would give an optimal enhancing result. The results of chitosans which have demonstrated efficient absorption enhancing activities were then compared with that of the reference enhancers.

Two types of cyclodextrin derivatives namely dimethyl-β-cyclodextrin (DM-β-CD) and hydroxypropyl-β-cyclodextrin (HP-β-CD), were used as the reference enhancers. The reasons for choosing them were described in Chapter IV. The sample solutions contained sCT (1.75 μM) with 5 % w/v of either DM-β-CD or HP-β-CD in isotonic phosphate buffer pH 7.4. The plasma calcium level was similarly measured. In addition, plasma level of immunoreactive sCT was also quantitated for DM-β-CD to determine its absolute bioavailability with respective to i.v. administration.

Analytical Method

Plasma Calcium Determination

The direct colorimetric method for determining plasma calcium with o-cresolphthalein complexone was introduced by Stern and Lewis (1957). Procedures for the determination of calcium were based on the

interaction of the cation with suitable chromogenic agents such as o-cresolphthalein complexone. This technique is very simple, direct and reliable.

Plasma calcium levels following nasal administration of sCT were determined colorimetrically with o-cresolphthalein complexone using a kit commercially available from Clinag (Bangkok, Thailand). The reagent contained an o-cresolphthalein complexone (phenolphthalein, 3′,3″ bis-{[bis-(carboxymethyl) amino] methyl}- 5′,5″-dimethyl) act as color reagent ,which specifically complexes with calcium to form a purple colored complex in alkaline environment that use the ethanolamine as buffer reagent. This purple colored complex, measured with a spectrophotometer at $\lambda = 570$ nm, is directly proportional to the calcium concentration in the sample. Standards representing equivalent calcium concentration of 2-12 mg/dl, are prepared in a similar manner. The concentration of the unknown is determined from a standard curve.

$$\text{Calcium} + \text{o-Cresolphthalein Complexone} \xrightarrow[\text{medium}]{\text{alkaline}} \text{Calcium--Cresolphthalein Complexone Complex (Purple Color)}$$

The assay procedures are as follows :

A: Set spectrophotometer wavelength at 570 nm and absorbance reading to zero with water as reference.

B: Set up a series of tubes for standard and sample.

C: Two milliliters of buffer agent and two milliliters of color agent (provided with the kit) was added to sample or standard tubes.

D: After mixing by vortex, the content of each tube was read within 30 min The first absorbance (A1) indicated the extent of calcium which may have contaminated the pipet or test tube.

E: Fifty microliter of sample or standards was individually added to the tubes.

F: Mixing by gentle inversion and incubate for 5 min. at room temperature and the second absorbance (A2) was measured at 570 nm by using water as reference. A2 was expressed as the absorbance of total calcium (the combination of calcium which contaminated the container (pipet, test tube) and calcium in plasma)

So the absorbance of calcium in plasma (A) = A2 - A1 .....(1)

Calculation of calcium concentration in the sample was as follows:

Calcium Concentration (mg/dl)

$$= \frac{\text{A sample}}{\text{A standard}} \times \text{Concentration of standard} \quad \text{.....(2)}$$

G: The observed changes in plasma calcium level were expressed as percent of the initial calcium value (0 min sample)

$$\text{or \% plasma calcium remaining at any time} = \frac{\text{A sample at time t}}{\text{A sample at time 0}} \times 100\ \% \quad \text{.....(3)}$$

where A = Absorbance of calcium in plasma measured at 570 nm.

Plasma sCT Determination

Plasma immunoreactive sCT was quantitated by radioimmunoassay using an RIA kit developed by Peninsula Laboratories (Belmont, CA.). This technique is essentially the double antibody assay and has a minimum detectable concentration ranging from 10 to 1280 pg/ml depending on the plasma sample size. The principle is based upon the ability of a limited quantity of antibody to bind a fixed amount of radiolabeled antigen. It is assumed that both the labeled and unlabeled antigens have the same affinity for the antibody resulting in competitive binding between the two species. Therefore, the extent of the binding of radiolabeled antigen to antibody will depend on the amount of both labeled and unlabeled antigens. The greater the amount of unlabeled antigen present in the test sample, the smaller is the percentage of the bound radiolabeled antigen detected by a gamma counter. Separation of the bound and free radiolabeled antigen is necessary in order to determine the quantity of the unlabeled antigen. This can be achieved by precipitating the antigen-antibody complexes by adding a second antibody directed toward the immunoglobulin present in the original antiserum. The quantity of unlabeled antigen in an unknown sample is then determined by comparing the radioactivity of the precipitate, after centrifugation, with the values established using known standards in the same assay system.

Briefly, the assay procedures are as follows :

A: Polystyrene disposable tubes (12 X 75 mm) were labeled with the following designations : Total Counts (TC), tubes 1 and 2 ; Non - Specific Binding (NSB), tubes 3 and 4 ; Total Binding (TB),

tubes 5 and 6 ; sCT Standards , tubes 7 through 22 ; tubes starting with number 23 and after were assigned for plasma samples and also prepared in duplicate.

B: Two hundred microliters of RIA buffer was added to TC tubes (tube 1& 2) and NSB tubes (tube 3 & 4), RIA buffer provided with the kit.

C: One hundred microliters of RIA buffer was added to TB tubes (tube 5 & 6).

D: One hundred microliters of each sCT standard (concentrations 10, 20, 40, 80, 160, 320, 640 and 1280 pg/ml) was added in duplicate to tubes 7 to 22. The standard solutions were also provided with the kit.

E: One hundred microliters of each plasma sample was added in duplicate to all tubes starting with number 23 and up.

F: One hundred microliters of primary antibody (rabbit anti-peptide serum) was added to all tubes except TC and NSB tubes.

G: All the tubes were gently vortexed, covered and incubated overnight for 16-24 hr at 4° C.

H: One hundred microliters of tracer (^{125}I-salmon calcitonin) solution was added to all tubes.

I: All the tubes were again vortexed, covered and incubated overnight for 16-24 hr at 4° C.

J: One hundred microliters of Goat Anti-Rabbit IgG serum (GARGG) was added to each tube in the RIA.

K: One hundred microliters of Normal Rabbit Serum (NRS) was added to each tube in the RIA.

L: Vortex the contents of each tube and allowed to stand at room temperature for 90 min.

M: Add five hundred microliters of RIA Buffer to each tube and vortex

the contents.

N: They were centrifuged at 3,000 rpm (approx. 1,700 x g) for 20 min at 4° C using Sigma 320 K refrigerated centrifuge.

O: The supernatant of each tube (except TC) was carefully decanted into a radioactive waste container.

P: The radioactivity in all tubes were counted for one min. using Gamatec II ™.

After the values in count- per- minute (CPM) were obtained for each tube, a correction for the nonspecific binding was made by subtracting the average NSB count from the average CPM of each tube to obtain average net CPM (Equation. 4). The percentage of bound radiolabeled antigen ($\%B/B_0$) was then determined from the following formula :

$$\text{Average net CPM} = \overline{\text{CPM}} - \overline{\text{NSB}} \quad \text{.....(4)}$$

The percentage of bound radiolabeled antigen ($\%B/B_0$) for the duplicate standards and unknown samples use the following calculation:

$$\%B/B_0 = [\text{net } \overline{\text{CPM}} \text{ std. or unknown} / \text{net } \overline{\text{CPM}}\ B_0] \times 100\ \% \text{... .(5)}$$

$$B_0 = \overline{\text{TB}} - \overline{\text{NSB}}$$

where $\overline{\text{CPM}}$ = average count per minute

$\overline{\text{NSB}}$ = average non-specific binding

$\overline{\text{TB}}$ = average total binding

% B/B_0 for each standard was then plotted as a function of standard concentration of unlabeled sCTon semilog graph paper.The concentration of sCT in the plasma samples could be read directly from the standard curve (n = 2 replications).

Data Analysis

Phamacodynamic Data Analysis

The plasma calcium lowering effect in the individual sCT- treated rat was indicated by the total percent decrease in plasma calcium level during 0-240 min (% D) as compared to the appropriate baseline group (no sCT and enhancer) was calculated by a modification of Hirai et al. (1981) using the following equation :

$$\%\,D = \frac{[\,\overline{AUC}_{baseline} - AUC_{sCT\ treated}\,]}{\overline{AUC}_{baseline}} \times 100\ \% \quad \text{.....(6)}$$

where $\overline{AUC}_{baseline}$ = average area under plasma calcium curve from 0-240 min after nasal administration of 0.15 M phosphate buffer at the corresponding pH to rats of the baseline group.

$$= \sum_{i=1 \rightarrow n} [\,(C_i + C_{i-1})/2\,]\,\Delta t \quad \text{.....(7)}$$

$AUC_{sCT\ treated}$ = area under the plasma calcium curve from 0-240 min after nasal administration of sCT (with or without enhancer) to the individual rat

% D = total percent decrease in plasma calcium

The areas under all the individual plasma calcium concentration versus time curves (AUC_{0-240}) were calculated by a linear trapezoidal rule from 0 to 240 min.

Pharmacokinetic Data Analysis

All samples with showed sCT serum concentrations below the limit of quantitation (10 pg/ml) were set to 0 pg/ml. Estimation of the area under the curves of plasma sCT concentration versus time were calculated using the trapezoidal rule from 0 to 180 min (AUC_{0-180}). The absolute and relative nasal bioavailability were calculated for each nasal preparation by comparing its AUC_{0-180} to that following intravenous injection of 0.15 IU/Kg sCT.

$$\%\ F_{abs} = \frac{AUC_{(in)} \times Dose_{(iv)}}{AUC_{(iv)} \times Dose_{(in)}} \times 100\ \% \quad \text{..... (8)}$$

$$\%\ F_{rel} = \frac{AUC_{in(\text{with enhancer})} \times Dose_{(iv)}}{AUC_{in(\text{no enhancer})} \times Dose_{(in)}} \times 100\ \% \quad \text{..... (9)}$$

where AUC = average area under the plasma sCT curve as defined above

$\%\ F_{abs}$ = percent of the absolute bioavailability

$\%\ F_{rel}$ = percent of the relative bioavailability

$Dose_{(iv)}$, $Dose_{(in)}$ = intravenous and intranasal dose of sCT in IU/Kg, respectively

Part II. Possible Inhibitory Effects of Chitosans on Nasal Proteolytic Enzyme Activities

Proteolytic activities of the enzymes in the absorptive membranes are perhaps the most important barriers and can significantly reduce the systemic bioavailability of the protein and peptide drugs. The mucosal membranes of the nasal cavity are known to have various types of peptidase and protease activities, including both exopeptidases and endopeptidases (Zhou, 1994). The delivery of proteins and peptides has been hindered by the peptidase and protease activity in the nasal mucosa. The predominant enzymes appear to be aminopeptidases among other exopeptidases and endopeptidases. Aminopeptidase activity in the nasal mucosa has been found to be similar to that of the ileal mucosa in its subcellular distribution (Sarkar, 1992). Specifically, almost half of the albino rabbit enzyme is membrane bound. It has yet to be determined whether the membrane-bound aminopeptidases are active enough to degrade completely most of the peptides and proteins and whether the cytosolic aminopeptidases are active enough to degrade the peptides and proteins that have escaped hydrolysis in the membrane. Since the major enzymes acting as an important barrier to absorption of peptides from the nasal mucosa are aminopeptidases, inhibition of these enzymes should improve the absorption of susceptible peptide and protein drugs.

Morimoto and co-workers (1995) demonstrated that the proteolytic activities in homogenates of the nasal mucosa tissues of the rat were high and found to be in the following order : leucine aminopeptidase

> dipeptidyl aminopeptidase > cathepsin > trypsin. Leucine aminopeptidase, thus, appears to be most abundant in the nasal mucosa. Interestingly, the inhibition of trypsin minimize activity was also found to sCT degradation even its the low extent (Moimoto et al., 1995). It may be assumed that trypsin could be the main cause for degradation of protein and peptide drugs such as sCT.

In vitro method can be used to evaluate the possible inhibitory effects of chitosans on proteolytic enzyme activity. The enzyme inhibition studies were performed with two types of enzymes. The endopeptidase trypsin was chosen as a representative of the nasal enzyme in the serine group. Moreover, the leucine aminopeptidase enzyme was also studied. It belongs to the group of exopeptidase and appears to be the major enzymatic barrier to nasal peptide drug delivery. Both enzymes are important in pharmaceutical field, since they are involved in the nasal degradation of many peptide and protein drugs especially sCT (Zhou, 1994).

The aim of this part was to determine the potential of chitosans in inhibiting the proteolytic enzyme activity in vitro by using trypsin and leucine aminopeptidase as the nasal enzyme models.

2.1 Degradation Studies with Trypsin (EC 3.4.21.4)

Principle

The determination of trypsin activity is carried out by the method of Erlanger (1961). Trypsin activity was determined with the chromogenic

substrate " N-α-benzoyl-DL-arginine-p-nitroanilide HCl " (BAPA) and the hydrolysis of this substrate as catalyzed by trypsin releases p-nitroaniline which is yellow in color and can be estimated colorimetrically at 410 nm.

Preparation of Solutions

Buffer Solution :

The buffer system used in all the enzyme inhibition experiments was a 0.15 M phosphate buffer , pH 6.0.

Substrate Stock Solution (1 mM) :

Degradation studies with trypsin were performed using N-α-benzoyl-DL-arginine-p-nitroanilide HCl (BAPA) as enzyme substrate. Its high activity and its indicator-like properties (i.e., the release of color upon hydrolysis) make it ideal for the detection or quantitative determination of trypsin in biological systems (Erlanger et al, 1961). The substrate concentration in this experiment was 1.0 mM, which had been shown in the preliminary experiment to provide sufficient substrate for maximal hydrolytic activity measurements. This is necessary to provide a meaningful comparison of the enzyme activities. A concentration of 1.0 mM of the substrate was prepared by dissolving 43.5 mg of BAPA in 1.0 ml of dimethylsulfoxide, and the solution was brought to 100 ml with the phosphate buffer (pH 6.0) . Care must be taken to dissolve all of the BAPA in dimethylsulfoxide (Be sure no crystals remain). The temperature of the stock solution was always kept at 37° C and freshly prepared.

Enzyme Solution (0.2 mg/ml) :

The experiments were performed using the commercial trypsin enzyme, purified from bovine pancreas (Sigma). Each mg of solid enzyme was equivalent to 8,060 BAEE units, where each BAEE unit will produce a ΔA_{253} nm of 0.001 per min at pH 7.6 and at a temperature of 25°C using BAEE as substrate (BAEE is an abbreviated name for N-α-benzoyl-DL-arginine-ethylester). The enzyme solutions at concentration of 0.2 mg/ml were prepared by first dissolving 10 mg of trypsin in 10 ml of 0.001 M HCl and then diluting with the phosphate buffer (pH 6.0) to the volume of 50 ml. The stock solution was stored at 5 °C.

Test Solutions :

The two chitosans (the free base and glutamate salt) were prepared at various concentrations (0.25, 0.50, 0.75, 1.00 and 1.25 % w/v) by dissolving each one in sufficient quantity of 1% v/v acetic acid solution and allowing them to swell for several hours. Then, they were dispersed in phosphate buffer (pH 6.0) to the desired concentrations. The pH values were checked and adjusted to 6.0 with either 1 N NaOH or 1 N HCl.

The specific inhibitor solution of trypsin, i.e. aprotinin, was used for comparison with chitosans with respect to their capacity to inhibit trypsin activity. Aprotinin, a bovine pancreatic kallikrein inhibitor, consists of a single-chain polypeptide containing 58 amino acid residues with molecular weight of 6,500. It has been used to inhibit plasmin, trypsin, chymotrypsin and various intracellular proteases (Zhou et al., 1991). The

inhibitor was obtained from bovine lung (Sigma) and its activity was 4.4 TIU/mg, i.e. each mg of solid is equivalent to 4.4 TIU. (One trypsin inhibitor unit (TIU) will decrease the activity of 2 trypsin unit by 50 % where one trypsin unit will hydrolyze 1.0 μmole of N-α-benzoyl-DL-arginine-p-nitroanilide HCl per min at pH 7.8 at 25 °C). The inhibitor solution at concentration of 0.2 mg/ml (equivalent to 0.88 TIU/ml) was prepared by dissolving 1 mg of aprotinin into 5 ml of the phosphate buffer to give the required concentration. The stock solution of the inhibitor was stored at 2 to 8 °C. For the control group, the phosphate buffer (pH 6.0) was used instead of the sample solutions at equal volume.

Calibration Curve of p-nitroaniline

Ten mg of p-nitroaniline was accurately weighed and dissolved in 5 ml of distilled water. The solution was then adjusted to 100 ml with the phosphate buffer (pH 6.0) and used as stock solution (0.1 mg/ml). The final pH of the stock was adjusted to the same as the buffer (pH 6.0) by adding of either 1 N hydrochloric acid or 1 N sodium hydroxide solution. The stock solution was pipetted at volumes of 1, 2, 3, 6, 9, 12, and 15 ml into a series of 50 ml volumetric flask and diluted to volume with the phosphate buffer (pH 6.0). The final concentration of each solution were 2, 4, 6, 12, 18, 24 and 30 mg/ml x 10^{-3}, respectively.

The absorbance of each known standard concentration was determined using UV-Visible spectrophotometer in a 1-cm cell at 410 nm. The spectrophotometer was set for zero absorbance with phosphate buffer, pH 6.0. Each concentration was determined in triplicate.

Analytical Method

Inhibitor activity was assayed by the loss in enzyme activity following the incubation times of enzyme with varying amounts of inhibitors. The conversion of N-α-benzoyl-DL-arginine-p-nitroanilide HCl (BAPA) by trypsin to p-nitroaniline was measured as follows : At first, 20 ml of the substrate (1 mM) was incorperated with 5 ml of each chitosans or aprotinin preparations, respectively. Each substrate hydrolysis experiment was started after adding 5 ml of trypsin and the test solution was incubated in a thermostatically controlled bath at 37° C. The experiments were allowed to run for 240 min. Each sample volume of 3.5 ml was withdrawn at predetermined time intervals (0, 10, 20, 30, 40, 60, 90, 120 and 240 min) and adding with 0.5 ml of 30% acetic acid to stop the trypsin activity. The final volume of each reaction mixture was 4 ml in each sampling. In the incubation mixture, the final concentration of the enzyme was 0.029 mg/ml. Controls were run simultaneously for every the inhibition experiment. The controls differed from the test solutions by in having inhibitor solution replaced with an equivalent amount of buffer. The quantity of p-nitroaniline was estimated spectrophotometrically at 410 nm. Summary of the analytical procedures is given in Table 5.

The inhibitory activity of the enzyme was determined by calculating the area under the metabolite (p-nitroaniline) concentration versus time curve up to 240 min, using the trapezoidal rule. and comparison with the control group.

Table 5 Sample solution prepared for experiment of Part II (2.1) : Inhibition of trypsin activity by chitosans and aprotinin

Order	Substances	Volume (ml)	
		Control (ml)	Test (ml)
1	Phosphate buffer (pH 6.0)	10.0	5.0
2	BAPA substrate solution (1 mM)	20.0	
3	Test solution (chitosans or aprotinin)	-	5.0
4	Trypsin solution (0.2 mg/ml)	5.0	5.0
Incubate at 37 C and sampling at 0,10,20,30,40,60,90,120 and 240 min (each 3.5 ml)			
5	Add 30 % Acetic acid	0.5	
Total volume of each sample prior to absorbance measurement		4.0	

2.2 Degradation Studies with Leucine Aminopeptidase (LAP, L-alkyl-peptide hydrolase, EC 3.4.11.2)

Principle

The method used was that developed by Goldberg and Rutenberg (1958) as modified by Takenaka and Takahashi (1962). L-leucyl-β - naphthylamide (L-Leu-β-NA) was selected as the substrate. Hydrolysis leads to the formation of β-naphthylamine which is subsequently diazotized and coupled with sodium nitrite and N-(1-naphthyl)-ethylenediamine to produce a highly colored compound which can be quantified by absorbance measurement at 580 nm. The triplicate spectrophotometric measurements were made for each point in this study.

LAP catalyzes the first of the following reactions:

$$\text{L-leucyl-β-naphthylamide} + H_2O \xrightarrow[H+]{\text{LAP}} \text{L-leucine} + \text{β-naphthylamine}$$

$$\text{β-naphthylamine} + NaNO_2 \rightarrow \text{diazo reagent}$$

$$\text{diazo reagent} + \text{N-(1-naphthyl)-ethylenediamine} \rightarrow \text{blue azo dye}$$

The dye has an absorption maximum at 580 nm. The absorbance of the dye produced is proportional to the β-naphthylamine concentration which, in turn, reflects the original LAP activity.

Preparation of Sample Solutions

Buffer Solution :

The buffer system used in all the enzyme inhibition experiments was a 0.15 M phosphate buffer , pH 6.0.

Substrate Stock Solution (0.2 mg/ml or 0.78 mM) :

L-leucyl-β-naphthylamide (L-Leu-β-NA) was used as a substrate for leucine aminopeptidase. Twenty mg of L-Leu-β-NA was dissolved in phosphate buffer and make up to 100 ml. The pH of the solution was adjusted to pH 6.0 with 1 N NaOH or 1 N HCl. The substrate solution was stored at temperature below 0^{o} C and shaken well after thawing.

Enzyme Solution (0.067 unit/ml) :

The leucine aminopeptidase (microsomal) enzyme, obtained from porcine kidney microsomes (Sigma), was prepared by dissolving 2.1 mg (equivalent to 25 unit) in 5 ml phosphate buffer (pH 6.0) to obtain a stock solution. Each mg solid was equivalent to 12 units, where each unit was defined as being capable of hydrolyzing 1.0 μmol of L-leucyl-p-nitroanilide to L-leucine and p-nitroaniline per min at pH 7.2 and 37^{o} C. A 75 fold of enzyme dilution was prepared by adding 0.1 ml of the stock enzyme solution to 7.4 ml of the phosphate buffer. The final concentration of the enzyme was 5.6×10^{-3} mg/ml (equivalent to 0.067 unit/ml). The solution was stored at 5 °C until use.

Test Solutions :

The inhibitory effect of chitosans on the LAP activity was studied at various concentration 0.25, 0.50, 0.75, 1.00 and 1.25 % w/v) and the enhancer solutions were prepared by the same method as described above (see **2.1**). The specific inhibitor solution of leucine aminopeptidase, namely bestatin, was used for comparison of their capacity to inhibit enzyme activity. Bestatin is a specific inhibitor of leucine aminopeptidase isolated from the culture filtrate of Streptomyces olivoreticuli (MD 976-C7). The structure of bestatin was elucidated to be [(2S,3R)-3-amino-2-hydroxy-4-phenylbutanoyl]-(S)-leucine. It show a strong inhibition of leucine aminopeptidase in a competitive manner (Suda et al., 1976). So in this study used its to be reference inhibitor.

Bestatin solution at concentration of 0.145 mM was prepared by dissolving 5 mg of bestatin in 10 ml of the same buffer and used as stock solution. Then 1 ml of the solution was pipetted into 10 ml volumetric flask and diluted to volume with the buffer. A suitable control without inhibitor was also set up. For the control group, 0.15 M phosphate buffer was used instead of the inhibitor/chitosan solutions at equal volume.

Sodium Nitrite Solution (0.2% w/v)

The solution is prepared by dissloving 2 mg of sodium nitrite in 1 ml of water. It should be prepared fresh daily.

Alcoholic Dye Base Solution

The solution is prepared by adding 110 ml of 95% ethyl alcohol to 55 mg of N-1-Naphthylethylenediamine and shake well. Stored at 2-8° C for several months. Discard when solution becomes colored, or if microbial growth becomes evident.

Calibration Curve of β-naphthylamine

The β-naphthylamine standard stock solution in buffer pH 6.0 contained 0.018 mg β-naphthylamine / ml. The solution was individually pipetted into six tubes with various volumes as indicated in columns 2 and 3 (Table 6). Appropriate volumes of 2 N HCl were added to each tube to make total volume of 1.5 ml. The preparations were analyzed as described in steps 4 to 7 in the analytical method. The final nominal concentration of β-naphthylamine in the reaction mixture after adding sodium nitrite, ammonium sulfamate and alcoholic dye base solution, were 0, 3.6, 7.2, 14.4, 21.6 and 28.8 mg/ml x 10^{-4}. Summary of the analytical procedures for standard is provided in Table 6.

The absorbance of the dye was determined in a 1-cm cell using spectrophotometer at 580 nm.

Table 6 Calibration solution of β-naphthylamine prepared for experiment Part II (2.2)

Tube No.	β-naphthylamine standard solution (ml)	2 N HCl (ml)	Sodium Nitrite (ml)	Ammonium Sulfamate (ml)	Alcoholic Dye base solution (ml)	Conc. of β-naphthylamine (mg/ml) x 10^4
1	0.0	1.5	0.5	1.0	2.0	0
2	0.1	1.4	,,	,,	,,	3.6
3	0.2	1.3	,,	,,	,,	7.2
4	0.4	1.1	,,	,,	,,	14.4
5	0.6	0.9	,,	,,	,,	21.6
6	0.8	0.7	,,	,,	,,	28.8

* Final volume of the reaction mixture for each standard conc. = 5.0 ml

Analytical Method

1. For test solution, 5 ml of L-leu-β-NA was mixed with the 5 ml of chitosan or inhibitor solution.
2. After adding 5 ml of leucine aminopeptidase enzyme, the reactions were allowed to run for 4 hrs at 37°C.
3. Samples of 2 ml each were withdrawn from the reaction mixture at predetermined times intervals (0,10,20,30,40,60,90,120 and 240 min) and quenched with 0.5 ml of 2 N HCl . The solution was mixed by gently shaking. The total volume in each withdrawn sample after quenching was 2.5 ml.

4. To each tube, add 0.5 ml sodium nitrite solution. Mix quickly and wait exactly 3 minutes at room temperature.

5. To each tube, add 1.0 ml ammonium sulfamate (provide in the kit). Mix. quickly and wait exactly 3 minutes at room temperature.

6. To each tube, add 2.0 ml alcoholic dye base solution. Mix and wait 45 ±10 minutes at room temperature. The final volume in each sample was 6.0 ml.

7. Transfer to cuvettes and read absorbance at 580 nm by using phosphate buffer as reference. The control solutions were also similarly prepared for every inhibition experiment with the exception that the inhibitor solution was replaced by an equal volume (5 ml) of buffer pH 6.0. Summary of the analytical procedures is given in Table 7.

Statistical Analysis

All data were expressed as mean ± SD. Statistical evaluation of the data was made by Student's t-test and analysis of variance (ANOVA) and by multiple comparison of the means using Duncan's test where appropriate. Differences between group means were judged significant at p-value < 0.05. The computation was performed using a statistical software package (SAS Inc.).

Table 7 Sample solution prepared for experiment of Part II (2.2) : Inhibition of LAP activity by chitosans and bestatin

Order	Reagent	Volume (ml)	
		Control (ml)	Test (ml)
1	Phosphate buffer (pH 6.0)	10.0	5.0
2	LAP substrate solution (7.8 mM)	5.0	
3	Test solution (chitosans or bestatin)	-	5.0
4	LAP solution (0.067 unit/ml)	5.0	
Incubate at 37 C and sampling at 0,10,20,30,40,60,90,120 and 240 min (each 2.0 ml)			
5	2N HCl	0.5	
6	Sodium nitrite solution	0.5	
7	Ammonium sulfamate solution	1.0	
8	Alcoholic dye base solution	2.0	
Mix and wait 45 ± 10 min at room temperature			
Total volume of each sample before measurement of absorbance		6.0	