Chapter 4

MATERIALS & METHODS

4.1 MATERIALS

All active materials and excipients were kindly supplied by Mercury Laboratories Ltd, Baroda along with certificate of Analysis. All other Chemicals and solvents were of analytical grades and purchased from S.d.fine-Chem Ltd, Mumbai.

Fungal alpha amylase [1:5555] I.P. Grade (Biocon, Bangalore)
Pepsin [1:3000] I.P. Grade (Biocon, Bangalore)
Papain I.P. Grade (Biocon, Bangalore)
Sucrose I.P. Grade (M.B.Sugars, Nasik)
Sodium methyl paraben I.P. Grade (Maruti Chemicals, Ahmedabad)
Sodium propyl paraben I.P. Grade (Maruti Chemicals, Ahmedabad)
Bronopol B.P. Grade (Jayco Chemical Industries, Mumbai)
Caramel colour I.P. Grade- Gem Flavours Pvt Ltd, Chennai)
Cardamom essence (Bush Boake Allen India Ltd, Mumbai)
Glycerin I.P. Grade (Gulshan polyols Ltd, Bharuch)
Polyethylene glycol 400 I.P. Grade (Laffans Petrochemicals Ltd, Ankleshwar)
Calcium chloride I.P. Grade (Shanpar Industries Pvt Ltd, Baroda)
Calcium gluconate I.P. Grade (Shanpar Industries Pvt Ltd, Baroda)
Calcium lactate I.P. Grade (Lactochem Ltd, Tamilnadu)
Calcium propionate A.R. Grade (S.d.fine-chem ltd, Mumbai)
Sodium chloride I.P. Grade- Shanpar Industries Pvt Ltd, Baroda)
Magnesium chloride I.P. Grade (Shanpar Industries Pvt Ltd, Baroda)
Sodium benzoate I.P. Grade (Shanpar Industries Pvt Ltd, Baroda)
Guar gum I.P. Grade (Drytech, Mumbai)
Colloidal silicon dioxide I.P. Grade (Degussa, Mumbai)
Quinoline yellow (ASIM Products, Mumbai)
Pineapple powder flavour (Bush Boake Allen India Ltd, Mumbai)
Dill oil B.P. Grade (Rakesh Products, Kanpur)
Anise oil B.P. Grade (Rakesh Products, Kanpur)
Caraway oil B.P. Grade (Rakesh Products, Kanpur)
Propylene glycol I.P. Grade (Dow Chemicals, Brazil)
Propyl gallate I.P. Grade (Camlin Ltd, Mumbai)
Sunset yellow (ASIM Products, Mumbai)
Lactose I.P. Grade (Lactose India, Baroda)

Talc I.P. Grade- Neelkanth Minechem, Jodhpur)

Beta-cyclodextrin A.R. Grade- S.d.fine-chem ltd, Mumbai)

Tris buffer A.R. Grade- S.d.fine-chem ltd, Mumbai)

Sorbitol I.P. Grade- Kashap Sweetners, Dhar)

Sorbitol 70 % solution I.P. Grade (Kashap Sweetners, Dhar)

Povidone K-30 I.P. Grade (BASF, USA)

Isopropyl alcohol I.P. Grade (Reliance Chemicals, Mumbai)

Magnesium stearate I.P. Grade (Komal Industries, Ankleshwar)

Croscarmellose sodium B.P. Grade (Rosswell Industries, Ahemdabad)

Cellulose acetate phthalate I.P. Grade (G.M.Chemicals, Mumbai)

Diethyl phthalate U.S.P. Grade (Nidilac Chemicals, Mumbai)

Methylene chloride I.P. Grade (GACL, Baroda)

Titanium dioxide I.P. Grade (Signet, Mumbai)

Gum acacia I.P. Grade (Drytech, Mumbai)

Calcium carbonate I.P. Grade (Neelkanth Minechem, Jodhpur)

Ethyl acetate A.R. Grade (S.d.fine-chem ltd, Mumbai)

White bees wax I.P. Grade (Waxoil, Bharuch)

Carnauba wax I.P. Grade (Waxoil, Bharuch)

Shellac I.P. Grade (Hindustan shellac, Mumbai)

Disodium hydrogen phosphate A.R. Grade (S.d.fine-chem ltd, Mumbai)

Potassium dihydrogen phosphate A.R. Grade (S.d.fine-chem ltd, Mumbai)

Sodium Acetate A.R. Grade (S.d.fine-chem ltd, Mumbai)

Soluble Starch A.R. Grade (E.Merck India Ltd, Mumbai)

Iodine solution (0.05 M) A.R. Grade (E.Merck India Ltd, Mumbai)

Glacial acetic acid A.R. Grade (S.d.fine-chem ltd, Mumbai)

Sodium Chloride A.R. Grade-S.d.fine-chem ltd, Mumbai)

Agar Powder (Himedia, Mumbai)

4.2 EQUIPMENT AND INSTRUMENTS

Spray-dryer LSD-48(Jay Instruments & Systems Pvt. Ltd., Mumbai)

Cone Blender (Pharmalab Engineering India Pvt. Ltd., Mumbai)

Planetary Mixer (Pharmalab Engineering India Pvt. Ltd., Mumbai)

Rotary Tabletting Machine, CMB3-16 (Cadmach Machinery Co.Pvt. Ltd., Ahmedabad)

Coating Pan 9 inches (Kamal Industries, Mumbai)

Spraying gun (Pilot, Mumbai)

Manual Capsule Filling M/C, MF-30 (Pam Pharmaceutical & Allied Machinery Co.Ltd., Mumbai)

Strong Cobb Hardness Tester, T-SHT 17 (Tab Machines, Mumbai)

Tablet Friability Tester (Magumps, Mumbai)

Bulk Density Apparatus (Campbell Electronics, Mumbai)

Magnestic stirrer, 2MLH (Remi Equipments, Mumbai)

Stirrer, RQ-123 (Remi Motors, Mumbai)

Heating Mantle (Jaymet Laboratories Equipments, Baroda)

Humidity Oven, NEC-210R10 (Newtronic Equipments Co. Pvt. Ltd., Mumbai)

Heating Oven (Jaymet Laboratories Equipments, Baroda)

Incubator, CIC-71 (Cintex Industrial Corporation, Mumbai)

Fridge (Whirlpool of India Ltd., Mumbai)

Digital Water Bath (Jaymet Laboratories Equipments, Baroda)

S.S.Sieves (Jaymet Laboratories Equipments, Baroda)

UV-Visible Spectrometer, PharmaSpec UV-1700 (Shimadzu Corporation, Japan)

Spectrofluorophotometer, RF – 540 (Shimadzu Corporation, Japan)

FTIR Spectrometer, FTIR-5300 (Jasco Corporation, USA)

NMR Spectrometer (300 MHz, Varian, USA)

Differential Scanning Calorimeter, TA-4000 series-DSC-30 Thermo analyser (Mettler-Toledo, Switzerland)

Scanning Electron Microscope, JSM-5610LV (JEOL, Japan)

Laser Diffraction Particle Size Analyzer, 2000 SM (Malvern Instruments Ltd, UK)

Electronic Balance, AUW 220 D (Shimadzu Corporation, Japan)

pH Meter, PICO (Labindia Instruments Pvt.Ltd., Mumbai)

Digital Tablet Dissolution Test Apparatus, Disso 2000 (Labindia Instruments Pvt.Ltd. Mumbai)

Automatic KF Titrator, CL-4885 (Chemito, Nashik)

Colorimeter 1007 (Bausch & Lomb, USA)

Disintegration Tester, ED-2L (Electrolab India, Mumbai)

4.3 ANALYTICAL METHODS

a) Estimation of fungal alpha amylase activity

Estimation of Raw material fungal alpha amylase was carried out by Indian Pharmacopoeial method [1].

Reagent

1. Sodium acetate
2. Soluble starch
3. 0.05 M Iodine
4. Glacial acetic acid
5. Sodium chloride

Preparation of acetate buffer pH 5

Acetate buffer pH 5 was prepared by dissolving 13.6 gm of sodium acetate and 6 ml of glacial acetic acid in sufficient water to produce 1000 ml.

Preparation of starch substrate

Soluble starch equivalent to 1 g of the dried substance was weighed and made slurry in 5 ml of water in beaker. Slurry was added to 75 ml of boiling water and rinsed the beaker twice with 5 ml of water, and added the washings to the hot starch solution and reboiled for 2 minutes with stirring continuously. Starch solution was cooled to 25°C and added 5 g of sodium chloride and sufficient water to produce 100 ml. Thereafter, 10 ml of starch solution was diluted to 100 ml with acetate buffer pH 5.

Preparation of 0.02 M Iodine

Iodine (0.05 M) ready solution was diluted to make 0.02 M iodine solution with distilled water.

Procedure

Fungal alpha amylase quantity equivalent to 100 Units of amylase activity was weighed accurately and triturated with 200 ml of acetate buffer pH 5 and added sufficient acetate buffer pH 5 to produce 1000 ml. From the above solution 10 ml was diluted to 100 ml with acetate buffer pH 5 to give the test solution and filtered (1 ml of the test solution should be capable of digesting about 10 mg of dry soluble maize or corn starch). Thereafter, 5 ml of starch substrate was added into each of six test-tubes without touching the sides of the test-tube. These test tubes were placed in a water-bath maintained at 40° ± 0.1°C. When the temperature of the solution in the tubes was reached to

40°C, the test solution 0.35 ml, 0.4 ml, 0.45 ml, 0.5 ml, 0.55 ml and 0.6 ml were added to each of the test-tubes marked 1 to 6 respectively and recorded the time of addition and mixed thoroughly and replaced the tubes in the water-bath. After exactly 60 minutes, tubes were removed and cooled rapidly in cold water. Thereafter, 0.05 ml of iodine solution was added to each tube and mixed well. The tube containing the lowest volume of test solution which did not show a bluish or violet tinge was noted and from this volume calculated the number of grams of dry soluble starch digested by 1 g of the substance being examined. This represents the number of units of amylase activity per gm.

b) **Estimation of fungal alpha amylase activity from formulations**

Estimation of fungal alpha amylase from formulation was done by Radial Diffusion method or called as cup plate method [2].

Reagent

1. Sodium acetate
2. Soluble starch
3. 0.05 M Iodine
4. Glacial acetic acid
5. Sodium chloride
6. Agar powder

Preparation of acetate buffer pH 5

Acetate buffer pH 5 was prepared by dissolving 13.6 gm of sodium acetate and 6 ml of glacial acetic acid in sufficient water to produce 1000 ml.

Standard preparation

Fungal alpha amylase (1:1200) working standard was weighed accurately (50 mg) in 100 ml volumetric flask and volume was made to 100 ml with acetate buffer pH 5. Thereafter, 5 ml of this solution was diluted to 50 ml with acetate buffer pH 5 (SH). To the 2 ml of above solution, added 6 ml acetate buffer pH 5 in a test tube (SL).

Sample preparation

Sample equivalent to 50 mg of fungal alpha amylase (1:1200) was weighed in 100 ml volumetric flask and volume was made to 100 ml with acetate buffer pH 5. Thereafter, 5 ml of this solution was diluted to 50 ml with acetate buffer

pH 5 (UH). To the 2 ml of above solution, added 6 ml acetate buffer pH 5 in a test tube (UL).

Preparation of substrate medium

Medium was prepared by accurately weighing 2.5 gm of soluble starch, 2.5 gm of agar powder and 0.5 gm of sodium chloride in 100 ml volumetric flask. About 50 ml of acetate buffer pH 5 was added and boiled to dissolve completely. The volume was made to 100 ml with acetate buffer pH 5 and cooled to approximately 45°C. Thereafter, 25 ml of above media was poured in Petri dish and allowed to solidify the media and such four plates were prepared. With the help of cork borer, four wells of about 8 mm diameter were prepared in each plate keeping adequate distance from each other.

Procedure

In each well of four plates, 0.1 ml of aliquot from the solution SH, SL, UH, UL were added carefully. The solution was allowed to diffuse into the plate completely and then incubated the plates for 24 hours at 37°C. Next day the plates were flooded with dilute iodine solution and colourless zones were seen around the well. The zone of starch digestion was measured with the help of zone reader in mm and percentage of fugal alpha amylase were calculated by the following formula

$$\% \text{ Potency} = \text{Antilog } (2 \pm a \log I) \quad \text{where}$$

I = dilution ratio

$$a = \frac{(UH + UL) - (SH + SL)}{(UH - UL) + (SH - SL)}$$

4.4 PREPARATION AND STABILITY STUDY OF FUNGAL ALPHA AMYLASE FORMULATIONS

Digestive enzyme preparations containing fungal alpha amylase and pepsin are available in market as oral liquid and oral drop formulations. These market preparations are not stable upon storage at room temperature and hence overages are normally added. Therefore the present study was undertaken to develop stable oral liquid and oral drop formulations for digestive disorders.

In present study, besides oral liquid and drops, proposed conventional formulations such as dry syrup, capsule; Novel formulations such as enterosoluble capsules, tablets, matrix tablets, dry syrup, pellets and oral liquid with cyclodextrin inclusion complex were prepared for better stability and efficacy.

4.4.1 ORAL LIQUID FORMULATIONS

4.4.1.1 Preformulation studies

a) Market sample study

Market survey was carried out to find the availability of different dosage form containing fungal alpha amylase and pepsin. Eight leading market samples of oral liquid containing fungal alpha amylase and pepsin were analysed for physical parameter and carried out the assay of fungal alpha amylase and pepsin. Fresh manufactured samples as well as near expiry samples were analysed to check the degradation pattern of enzymes.

b) Effect of pH on fungal alpha amylase activity

Fungal alpha amylase was dissolved at concentration 10 mg/ml in buffered solution having pH 1 to pH 9 and kept at room temperature for 24 hours. After 24 hours, the activity of fungal alpha amylase remained was analysed.

c) Effect of temperature on fungal alpha amylase activity

1. Solid state

Fungal alpha amylase [1:5555] powder was kept at different temperature such as refrigerated condition (2- 8°C), 30 °C, 45 °C and 40°C with 75 % RH for three month in open glass vial. Fungal alpha amylase activity and moisture content was estimated at an interval of 0, 1, 2 and 3 months.

2. Liquid state

Fungal alpha amylase [1:5555] was dissolved in mixed phosphate buffer pH 7 at 2.16 mg/ml concentration and kept at different temperature such as refrigerated condition (2- 8°C), 30 °C, and 45 °C for three month in glass bottle. These solutions were preserved with addition of sodium methyl paraben 0.2 % w/v and sodium propyl paraben 0.02 % w/v. Fungal alpha amylase activity was estimated at an interval of 0, 1, 2 and 3 months.

d) Effect of salts on fungal alpha amylase activity

Fungal alpha amylase [1:5555] was dissolved in mixed phosphate buffer pH 7 at 2.16 mg/ml concentration and added separately calcium chloride, magnesium chloride and sodium chloride at concentration of 0.4 mg/ml. These solutions were preserved with addition of sodium methyl paraben 0.2 % w/v and sodium propyl paraben 0.02 % w/v. These solutions were kept at 30 °C and 45 °C for one month in glass bottle. Fungal alpha amylase activity was estimated initially and after one month.

e) Enzyme-Cofactor interaction

1. Characterization

Fungal alpha amylase [1:5555] was dissolved in mixed phosphate buffer pH 7 at 2.16 mg/ml concentration and added cofactor calcium chloride at concentration of 0.4 mg/ml at room temperature. This solution was subjected to following analysis and compared with fungal alpha amylase [1:5555] in mixed phosphate buffer pH 7 at 2.16 mg/ml concentration.

Ultra-Voilet (UV) spectroscopy studies

UV spectral measurements were carried out using UV Spectrometer in absorbance range between 190 and 400 nm.

Fluorescence spectroscopy studies

Fluorescence spectral measurements were performed using Spectrofluorophotometer. Fluorescence spectra of the above solution were taken at 425 nm excitation wavelength and emission wavelength in the range 200- 500 nm.

Fourier Transform Infrared (FTIR) spectroscopy

FTIR spectral measurements were performed using FTIR Spectrometer. The above solution was placed in liquid cell and Spectra were taken in the range 4000- 400 cm^{-1} and the buffer contribution was subtracted.

2. Effects of disodium EDTA

Fungal alpha amylase [1:5555] was dissolved in mixed phosphate buffer pH 7 at 2.16 mg/ml concentration and added 10 mM of disodium EDTA at room temperature. After four hours, fungal alpha amylase activity was estimated.

4.4.1.2 Preparation of oral liquid formulation

a) Formulation

Oral liquid formulations were prepared at dose of fungal alpha amylase IP (1:1200) 50 mg and pepsin IP (1:3000) 10 mg per 5 ml.

In the present investigation, three different kind of oral liquid formulations were prepared at different pH without addition of cofactor or refolding aids; with addition of different concentrations of cofactors such as calcium chloride, calcium gluconate, calcium lactate, calcium propionate and with the addition of refolding aids such as sorbitol, polyethylene glycol 400, glycerin.

Syrup was prepared by dissolving weighed amount of sucrose in boiling purified water and preservatives sodium methyl paraben, sodium propyl paraben and bronopol were added. The syrup was cooled to room temperature and pH was adjusted to desired pH with 10 % citric acid. Then fungal alpha amylase was dissolved in purified water along with or without cofactor or refolding aid and added to syrup. Pepsin was dissolved in purified water and added to syrup under stirring. Colour and flavour were added to syrup. Syrup pH was adjusted to desired pH with 10 % citric acid or with mixed phosphate buffer. Finally volume of syrup was made with purified water and syrup was filtered through nylon cloth and filled in amber glass bottle.

These formulations were subjected to three month accelerated stability study at 45°C, 30°C, refrigerated condition (2-8°C) and enzyme activity were estimated at an interval of 0,1,2,3 months.

b) Preparation of oral liquid formulation without addition of cofactor and refolding aid

The batches of oral liquid formulations were prepared as per the method described in section 4.4.1.2.a. Before addition of enzymes, Batch Co-L-1 syrup pH was set to 5 and pH 7 for Batch Co-L-2. The composition of the Batches Co-L-1 and Co-L-2 is shown in Table 4.1.

Table 4.1 Oral liquid formulation Batch Co-L-1 and Co-L-2 without addition of cofactor and refolding aid

Ingredients	**Co-L-1**	**Co-L-2**
Fungal alpha amylase [1:5555]	1.62 gm	1.62 gm
Pepsin [1:3000]	1.5 gm	1.5 gm
Sucrose	275.0 gm	275.0 gm
Sodium methyl paraben	1.0 gm	1.0 gm
Sodium propyl paraben	0.1 gm	0.1 gm
Bronopol	0.5 gm	0.5 gm
Caramel colour	0.025 gm	0.025 gm
Cardamom essence	0.5 gm	0.5 gm
Purified water q.s.to	500.0 ml	500.0ml
Final pH adjusted to	5.0	7.0

c) Oral liquid formulation with refolding aid

The batches of oral liquid formulations were prepared as per the method described in section 4.4.1.2.a. Before addition of enzymes, pH was set to 7 and Batch Co-L-3 and Co-L-4 where enzymes added along with refolding aids such as glycerin or polyethylene glycol 400. In Batch Co-L-5 sugar syrup is not prepared; all additions were made in sorbitol 70 % solution as refolding aid. The composition of the Batches Co-L-3 to Co-L-5 is shown in Table 4.2.

Table 4.2 Oral liquid formulation Batch Co-L-3 to Co-L-5 with addition of refolding aid

Ingredients	**Co-L-3**	**Co-L-4**	**Co-L-5**
Fungal alpha amylase [1:5555]	1.62 gm	1.62 gm	1.62 gm
Pepsin [1:3000]	1.5 gm	1.5 gm	1.5 gm
Sucrose	275.0 gm	275.0 gm	----
Glycerin	25.0 ml	----	----
Polyethylene glycol 400	----	15.0 ml	----
Sodium methyl paraben	1.0 gm	1.0 gm	1.0 gm
Sodium propyl paraben	0.1 gm	0.1 gm	0.1 gm
Bronopol	0.5 gm	0.5 gm	0.5 gm
Caramel colour	0.025 gm	0.025 gm	0.025 gm
Cardamom essence	0.5 gm	0.5 gm	0.5 gm
Purified water q.s.to	500.0 ml	500.0 ml	----
Sorbitol 70 % solution q.s. to	----	----	500.0 ml
pH adjusted to	7.0	7.0	7.0

d) Oral liquid formulation with cofactor

The batches of oral liquid formulations were prepared as per the method described in section 4.4.1.2.a. Oral liquid formulations were prepared with different cofactor calcium chloride, calcium gluconate, calcium lactate, calcium propionate. Before addition of enzymes, syrup pH was set to the desired value.

1) With calcium chloride

The composition of the Batches Co-L-6 to Co-L-9 is shown in Table 4.3.

Table 4.3 Oral liquid formulation with cofactor calcium chloride

Ingredients	**Co-L-6**	**Co-L-7**	**Co-L-8**	**Co-L-9**
Fungal alpha amylase [1:5555]	1.62 gm	1.62 gm	1.62 gm	1.62 gm
Pepsin [1:3000]	1.5 gm	1.5 gm	1.5 gm	1.5 gm
Sucrose	275.0 gm	275.0 gm	275.0 gm	275.0 gm
Sodium methyl paraben	1.0 gm	1.0 gm	1.0 gm	1.0 gm
Sodium propyl paraben	0.1 gm	0.1 gm	0.1 gm	0.1 gm
Bronopol	0.5 gm	0.5 gm	0.5 gm	0.5 gm
Calcium chloride	0.1 gm	0.2 gm	0.3 gm	0.2 gm
Caramel colour	0.025 gm	0.025 gm	0.025 gm	0.025 gm
Cardamom essence	0.5 gm	0.5 gm	0.5 gm	0.5 gm
Purified water q.s.to	500.0 ml	500.0ml	500.0 ml	500.0ml
pH adjusted to	5.0	5.0	5.0	7.0

2) With calcium gluconate

The composition of the Batches Co-L-10 to Co-L-13 is depicted in Table 4.4.

Table 4.4 Oral liquid formulation with cofactor calcium gluconate

Ingredients	**Co-L- 10**	**Co-L- 11**	**Co-L- 12**	**Co-L- 13**
Fungal alpha amylase [1:5555]	1.62 gm	1.62 gm	1.62 gm	1.62 gm
Pepsin [1:3000]	1.5 gm	1.5 gm	1.5 gm	1.5 gm
Sucrose	275.0 gm	275.0 gm	275.0 gm	275.0 gm
Sodium methyl paraben	1.0 gm	1.0 gm	1.0 gm	1.0 gm
Sodium propyl paraben	0.1 gm	0.1 gm	0.1 gm	0.1 gm
Bronopol	0.5 gm	0.5 gm	0.5 gm	0.5 gm
Calcium gluconate	0.1 gm	0.2 gm	0.3 gm	0.2 gm
Caramel colour	0.025 gm	0.025 gm	0.025 gm	0.025 gm
Cardamom essence	0.5 gm	0.5 gm	0.5 gm	0.5 gm
Purified water q.s.to	500.0 ml	500.0ml	500.0 ml	500.0ml
pH adjusted to	5.0	5.0	5.0	7.0

3) With calcium lactate

The composition of the Batches Co-L-14 to Co-L-17 is shown in Table 4.5.

Table 4.5 Oral liquid formulation with cofactor calcium lactate

Ingredients	**Co-L- 14**	**Co-L- 15**	**Co-L- 16**	**Co-L- 17**
Fungal alpha amylase [1:5555]	1.62 gm	1.62 gm	1.62 gm	1.62 gm
Pepsin [1:3000]	1.5 gm	1.5 gm	1.5 gm	1.5 gm
Sucrose	275.0 gm	275.0 gm	275.0 gm	275.0 gm
Sodium methyl paraben	1.0 gm	1.0 gm	1.0 gm	1.0 gm
Sodium propyl paraben	0.1 gm	0.1 gm	0.1 gm	0.1 gm
Bronopol	0.5 gm	0.5 gm	0.5 gm	0.5 gm
Calcium lactate	0.1 gm	0.2 gm	0.3 gm	0.2 gm
Caramel colour	0.025 gm	0.025 gm	0.025 gm	0.025 gm
Cardamom essence	0.5 gm	0.5 gm	0.5 gm	0.5 gm
Purified water q.s.to	500.0 ml	500.0ml	500.0 ml	500.0ml
pH adjusted to	5.0	5.0	5.0	7.0

4) With calcium propionate

The composition of the Batches Co-L-18 to Co-L-21 is shown in Table 4.6.

Table 4.6 Oral liquid formulation with cofactor calcium propionate

Ingredients	**Co-L- 18**	**Co-L- 19**	**Co-L- 20**	**Co-L- 21**
Fungal alpha amylase [1:5555]	1.62 gm	1.62 gm	1.62 gm	1.62 gm
Pepsin [1:3000]	1.5 gm	1.5 gm	1.5 gm	1.5 gm
Sucrose	275.0 gm	275.0 gm	275.0 gm	275.0 gm
Sodium methyl paraben	1.0 gm	1.0 gm	1.0 gm	1.0 gm
Sodium propyl paraben	0.1 gm	0.1 gm	0.1 gm	0.1 gm
Bronopol	0.5 gm	0.5 gm	0.5 gm	0.5 gm
Calcium propionate	0.1 gm	0.2 gm	0.3 gm	0.2 gm
Caramel colour	0.025 gm	0.025 gm	0.025 gm	0.025 gm
Cardamom essence	0.5 gm	0.5 gm	0.5 gm	0.5 gm
Purified water q.s.to	500.0 ml	500.0ml	500.0 ml	500.0ml
pH adjusted to	5.0	5.0	5.0	7.0

4.4.1.3 Evaluation of the oral liquid formulations

Following parameters were evaluated

1) Description

Oral liquid was examined for colour, clarity, flavour.

2) pH [3]

pH was measured by using pH meter at a temperature of 25° ± 2°C.

3) Specific gravity [4]

Specific gravity was determined by finding out the ratio of the weight of sample in air at 25°C to the equal volume of water at the same temperature .

4] Assay of fungal alpha amylase

Activity of fungal alpha amylase was determined by Radial diffusion method as described in section 4.3b.

5] Assay of pepsin [5]

Activity of pepsin was determined by method given in Indian Pharmacopoeia.

4.4.2 DRY SYRUP FORMULATIONS

4.4.2.1 Preparation of dry syrup formulations

Dry syrup formulations were prepared at dose of fungal alpha amylase IP (1:1200) 50 mg and pepsin IP (1:3000) 10 mg per 5 ml of reconstituted syrup.

The oral dry syrup was prepared by two methods, by wet granulation and by dry mixing. In wet granulation method, weighed quantity of fungal alpha amylase and pepsin were sifted through 40 # sieve and mix homogenously. The enzyme blend was mixed with sweetening agent sucrose of 40 # size in cone blender. Thereafter preservative and cofactor were dissolved in purified water and sucrose as binding agent was added and dissolved completely by heating at 95° – 100 °C. The binder solution was cooled to room temperature and colour was dissolved into it. The binder solution was added to enzyme blend and mixed to form coherent mass in planetary mixer. The wet mass was passed through 8 # sieve and dried at 40°-45°C till moisture content is less than 2 %. Thereafter the dried granules were passed through 18 # sieve. Suspending agent, glidant and powder flavour were added to dried granules passing through 40 # sieve and mixed in cone blender. Thereafter, 8 gm of dry syrup were filled into 30 ml marking glass bottles. The composition of the Batches Co-DS-1 and Co-DS-2 is shown in Table 4.7.

The dry mixing method involved the step of mixing of all ingredients in geometric fashion by passing through 40 # sieve in cone blender. Thereafter, 8 gm of dry syrup were filled into 30 ml marking glass bottles. The composition of the Batches Co-DS-3 and Co-DS-4 is given in Table 4.8.

These formulations were subjected to accelerated stability study at 45°C, 30°C, and 40°C with 75 % RH and enzyme activity was estimated at an interval of 0,1,2,3 months. Reconstituted syrup was studied for 15 days at 30°C.

Table 4.7 Dry syrup formulation Batch Co-DS-1 and Co-DS-2 prepared by wet granulation method.

Ingredients	**Co-DS-1**	**Co-DS-2**
Fungal alpha amylase [1:5555]	1.944 gm	1.944 gm
Pepsin [1:3000]	1.8 gm	1.8 gm
Sucrose	143.226 gm	143.226 gm
Sucrose (as binder)	8.0 gm	8.0 gm
Sodium benzoate	0.24 gm	0.24 gm
Calcium chloride	----	0.24 gm
Quinoline yellow colour	0.05 gm	0.05 gm
Guar gum	1.0 gm	1.0 gm
Colloidal silicon dioxide	0.5 gm	0.5 gm
Pineapple powder flavour	3.0 gm	3.0 gm
Total weight	160.0 gm	160.0 gm

Table 4.8 Dry syrup formulation Batch Co-DS-3 and Co-DS-4 prepared by dry mixing method.

Ingredients	**Co-DS-3**	**Co-DS-4**
Fungal alpha amylase [1:5555]	1.944 gm	1.944 gm
Pepsin [1:3000]	1.8 gm	1.8 gm
Sucrose	151.226 gm	151.226 gm
Sodium benzoate	0.24 gm	0.24 gm
Calcium chloride	----	0.24 gm
Quinoline yellow colour	0.05 gm	0.05 gm
Guar gum	1.0 gm	1.0 gm
Colloidal silicon dioxide	0.5 gm	0.5 gm
Pineapple powder flavour	3.0 gm	3.0 gm
Total weight	160.0 gm	160.0 gm

4.4.2.2 Evaluation of the dry syrup formulations

a) Dry syrup blend evaluation

Following parameters were evaluated

1) Description

Oral dry syrup was examined for colour, flavour, powder flow.

2) Bulk density [6]

Bulk density was measured by introducing weighed quantity of sample (M) into dry measuring cylinder and without compacting, read the unsettled apparent volume, V_0, to the nearest graduated unit. The bulk density was calculated in gm/ml by following formula

$$(M)/ (V_0)$$

3] Tapped density [7]

Tapped density was measured by mechanically tapping the weighed quantity of sample (M) into dry measuring cylinder with the help of Bulk density apparatus and tapped for initially 500 times and measured the tapped volume. Repeated the tapping an additional 750 times and measured the tapped volume to nearest graduated unit. If the difference between two volumes is less than 2 % then final tapped volume noted as V_f and if not, then again repeated for 1250 tapping until the difference between succeeding measurements is less than 2 %. The tapped density was calculated in gm/ml by following formula

$$(M) / (V_f)$$

4] Compressibility index (CI) [8]

The compressibility index is the measure of the propensity of a powder to be compressed. As such it is the measure of the relative importance of interparticulate interactions. In free flowing powder, the bulk and tapped densities are closer in value. For poor flowing materials, there are frequently greater interparticulate interactions and a greater difference between the bulk and tapped densities are observed. These differences are reflected in Compressibility index, which is calculated by following formula

$$\frac{100\ (V_0 - V_f)}{V_0}$$

5) Angle of repose [9]

Angle of repose was measured by fixed funnel and free standing cone method which employed a funnel that was secured with tips at a given height, H, above the graph paper that was placed on a flat horizontal surface. The sample was carefully poured through the funnel until the apex of the conical pile just touched the tip of the funnel. Then radius of the base of the conical pile, R, was measured. The angle of repose (α) was calculated by following formula

$$\tan \alpha = \frac{H}{R}$$

6] Moisture content [10]

Moisture content was determined by automatic Karl Fischer titrator which is direct titration method. The dehydrated methanol was introduced into the titration vessel and titrated to the electrometric end point with the Karl

Fischer reagent. Accurately weighed sample was transferred to the titration vessel and stirred for 1 minute and titrated again to the electrometric end point using the Karl Fischer reagent. The water content of the sample, in mg is given by the expression $S \times F$, in which S is the volume, in ml of the Karl Fischer reagent used to titrate the sample and F is the water equivalent factor.

Water equivalent factor was determined by placing about 36 ml of dehydrated methanol in the titration vessel and added sufficient Karl Fischer reagent to give the characteristics end-point. Accurately weighed sodium tartrate was added quickly and titrated to the endpoint. The water equivalence factor, F, in mg of water per ml of the reagent is given by the expression 0.1566 (w)/(v), where w is the weight, in mg of the sodium tartrate and v is the volume, in ml, of the reagent required.

7] Assay of fungal alpha amylase

Activity of fungal alpha amylase was determined by Radial diffusion method as described in section 4.3b.

8] Assay of pepsin [5]

Activity of pepsin was determined by method given in Indian Pharmacopoeia.

b) Evaluation of the reconstituted dry syrup formulations

Following parameters were evaluated as described in section 4.4.1.3

1) Description
2) pH
3) Specific Gravity
4] Assay of fungal alpha amylase
5] Assay of pepsin

4.4.3 ORAL DROP FORMULATIONS

4.4.3.1 Preparation of oral drop formulations

Oral drop formulations were prepared at dose of fungal alpha amylase IP (1:800) 20 mg, papain IP 10 mg along with dill oil BP 2 mg, anise oil BP 2 mg and caraway oil BP 2 mg per ml.

Syrup was prepared by dissolving weighed amount of sucrose in boiling purified water and preservatives sodium methyl paraben and sodium propyl paraben were added. The syrup was cooled to room temperature. Syrup pH was adjusted to desired pH with 10 % citric acid. Caraway oil, dill oil and anise oil together were dissolved completely in propylene glycol under continuous stirring and blend was added to bulk syrup under stirring. Then fungal alpha amylase was dissolved in purified water along with or without cofactor and added to syrup. Papain was dissolved in purified water and added to syrup under stirring. Glycerin was added to the bulk syrup under stirring. Antioxidant propyl gallate was dissolved in propylene glycol and added to bulk syrup under stirring. Colour was dissolved in Purified water and added to the bulk under stirring. Syrup pH was adjusted to desired pH with 10 % citric acid or mixed phosphate buffer. Finally volume of syrup was made with purified water and syrup was filtered through nylon cloth and filled in amber glass bottle. The composition of the Batches Co-D-1 to Co-D-4 is shown in Table 4.9.

These formulations were subjected to three month accelerated stability study at 45°C, 30°C, refrigerated condition (2-8°C) and enzyme activity were estimated at an interval of 0,1,2,3 months.

Table 4.9 Oral drop formulations Batch Co-D-1 to Co-D-4

Ingredients	**Co-D-1**	**Co-D-2**	**Co-D-3**	**Co-D-4**
Fungal alpha amylase [1:5555]	2.16 gm	2.16 gm	2.16 gm	2.16 gm
Papain	7.5 gm	7.5 gm	7.5 gm	7.5 gm
Dill oil	1.0 gm	1.0 gm	1.0 gm	1.0 gm
Anise oil	1.0 gm	1.0 gm	1.0 gm	1.0 gm
Caraway oil	1.0 gm	1.0 gm	1.0 gm	1.0 gm
Propylene glycol	30 ml	30 ml	30 ml	30 ml
Sucrose	185.0 gm	185.0 gm	185.0 gm	185.0 gm
Sodium methyl paraben	1.0 gm	1.0 gm	1.0 gm	1.0 gm
Sodium propyl paraben	0.1 gm	0.1 gm	0.1 gm	0.1 gm
Propyl gallate	0.5 gm	0.5 gm	0.5 gm	0.5 gm
Glycerin	50.0 ml	50.0 ml	50.0 ml	50.0 ml
Calcium chloride	----	0.2 gm	0.3 gm	0.4 gm
Sunset yellow colour	0.025 gm	0.025 gm	0.025 gm	0.025 gm
Purified water q.s.to	500.0 ml	500.0ml	500.0 ml	500.0ml
pH adjusted to	7.0	7.0	7.0	7.0

4.4.3.2 Evaluation of the oral drop formulations

Following parameters were evaluated as described in section 4.4.1.3

1) Description

2) pH

3) Specific gravity

4] Assay of fungal alpha amylase

5] Assay of papain [11]

Papain activity was estimated by Indian Pharmacopoeial method.

4.4.4 HARD GELATIN CAPSULE FORMULATIONS

4.4.4.1 Preparation of hard gelatin capsule formulations

Capsule formulations were prepared at dose of fungal alpha amylase IP (1:1200) 50 mg and pepsin IP (1:3000) 10 mg per Capsule. Capsule formulations were prepared by dry mixing method. Fungal alpha amylase and cofactor were sifted through 40 # sieve and mixed for 5 minutes. Pepsin and lactose were passed through 40 # sieve and added to fungal alpha amylase blend and mixed for 15 minutes. Glidant was passed through 60 # sieve and added to above blend and mixed for 5 minutes. Capsules were filled at an average weight of 130 mg in ivory-ivory size "3" hard gelatin capsule by manual capsule filling machine. Capsules were polished and packed in glass bottle. The composition of the Batches Co-C-1 and Co-C-2 is shown in Table 4.10. These formulations were subjected to accelerated stability study at 45°C, 30°C, and 40°C with 75 % RH and enzyme activity estimated at an interval of 0,1,2,3 months.

Table 4.10 Hard gelatin capsule formulation Batch Co-C-1 and Co-C-2

Ingredients	**Co-C-1**	**Co-C-2**
Fungal alpha amylase [1:5555]	4.860 gm	4.860 gm
Pepsin [1:3000]	4.5 gm	4.5 gm
Calcium chloride	----	0.6 gm
Lactose	29.25 gm	28.65 gm
Talc	0.39 gm	0.39 gm
Total weight	39.0 gm	39.0 gm
Net content per capsule	130.0 mg	130.0 mg

4.4.4.2 Evaluation of the hard gelatin capsules

a) Evaluation of capsule blend

Following parameters were evaluated as described in 4.4.2.2

1) Description

Capsule blend was examined for colour, flow of powder

2] Bulk density

3] Tapped density

4] Compressibility index [CI]

5] Angle of repose

6] Moisture content

b) Evaluation of the hard gelatin capsules

Following parameters were evaluated

1) Description

Hard gelatin capsules were examined for colour and size of capsule, powder content colour.

2) Average net content per capsule

Twenty intact capsules were weighed, opened and removed the contents as completely as possible. Empty shells were weighed and determined the weight of content as the difference between two weighing. Thereafter, averages of net content were determined.

3) Average weight of intact capsule

Twenty intact capsules were weighed and average was determined.

4) Uniformity of weight [12]

Twenty intact capsules were weighed, opened and removed the contents as completely as possible. Empty shells were weighed and determined the weight of content as the difference between two weighing. Average weight was determined and percentage deviation from average weight was determined for individual capsule and compared the results with limits given in Indian pharmacopoeia as not more than two of the individual weights should deviate from the average weight by more than the percentage deviation and none should deviate by more than twice that percentage. Percentage deviation limit 10 % is given for less than 300 mg average weight of capsule content and 7.5 % for 300 mg or more average weight of capsule content.

5) Locking length

Twenty intact capsules length was measured and average was determined. The limit 15.8 mm ± 0.4 mm is given by the manufacturer.

6) Disintegration test [13]

Disintegration test was carried by using disintegration tester and water was used as medium. One capsule was introduced into each tube and disc added to each tube. The assembly was suspended into the glass beaker containing water and operated the apparatus till all capsules were disintegrated. Indian pharmacopoeia has given the disintegration time limit as 30 minutes for hard gelatin capsules.

7] Moisture content

Moisture content was determined as described in the section 4.4.2.2

8] Assay of fungal alpha amylase

Activity of fungal alpha amylase was determined by Radial diffusion method as described in section 5.3.

9] Assay of pepsin [5]

Activity of pepsin was determined by method given in Indian Pharmacopoeia.

4.4.5 ENTEROSOLUBLE FORMULATIONS

4.4.5.1 Preformulation studies

a) In-vitro stability studies of fungal alpha amylase

In–vitro stability of fungal alpha amylase were carried out at different pH as following

1) Stability at pH 1.2

Fungal alpha amylase as it is in powder form, in complex form with beta-cyclodextrin at 3:1 [beta-cyclodextrin: fungal alpha amylase] molecular ratio, and with 0.002 mg of calcium chloride were treated separately at concentration 50 mg per 900 ml of 0.1 N hydrochloric acid having pH 1.2 in Indian Pharmacopoeial dissolution apparatus type I (Paddle) at 37 °C ± 0.2 °C with 50 rpm speed. At an interval of one hour, 1 ml sample was drawn and enzyme activity was estimated.

2) Stability at different pH 1.2 – 5.0 – 6.8

Fungal alpha amylase as it is in powder form, in complex form with beta-cyclodextrin at 3:1 [beta-cyclodextrin: fungal alpha amylase] molecular ratio, and with 0.002 mg of calcium chloride were treated separately at concentration of 50 mg per 900 ml dissolution medium. This study was carried out in Indian Pharmacopoeia dissolution apparatus type I (paddle) at 37 °C ± 0.2 °C with 50 rpm speed. Initially dissolution was carried out at pH 1.2 for one hour and thereafter, dissolution medium pH was set to 5 by sodium acetate and dissolution was carried out for one hour. Again this dissolution medium pH was set to 6.8 by sodium acetate and dissolution was carried out for one hour. At every hour interval, 1 ml sample was drawn and fungal alpha amylase activity was estimated.

Similarly following studies were carried out,

3) Stability at different pH 3.0 – 5.0 – 6.8

4) Stability at different pH 4.0 – 5.0 – 6.8

5) Stability at different pH 5.0 – 6.8

b) Fungal alpha amylase market sample in-vitro stability studies

Oral liquid Batch Co-L-9 and eight leading market samples of oral liquid containing fungal alpha amylase and pepsin were analysed for in-vitro stability. This study was carried out at concentration of 50 mg per 900 ml of dissolution medium in Indian Pharmacopoeial dissolution apparatus type I (Paddle) at 37 °C ± 0.2 °C with 50 rpm speed. Initially dissolution was carried out at pH 3 for one hour and then dissolution medium pH was set to 6.8 by sodium acetate and dissolution was carried out for one hour. At every hour interval, 1 ml sample was drawn and fungal alpha amylase activity was estimated.

4.4.5.2 Preparation of enterosoluble formulations

These formulations were designed as targeted drug delivery system where pepsin was targeted to release in the acidic environment of stomach and fungal alpha amylase was targeted to release in the higher pH environment of the intestinal fluid. Fungal alpha amylase was protected from adverse effect of acidic condition with the help of enteric polymer cellulose acetate phthalate which is soluble above pH 6 and delivers the enzyme at higher pH environment of the intestinal fluid.

In the present study, four different kinds of formulation were prepared as enteric sugar coated tablet, enteric matrix sugar coated tablets, pellets formulation and enteric coated fungal alpha amylase powder. Enteric coated fungal alpha amylase powder further incorporated into tablets, dry syrup and capsules.

All enterosoluble formulations were subjected to accelerated stability study at 45°C, 30°C, and 40°C with 75 % RH and enzyme activity was estimated at an interval of 0,1,2,3 months.

a) Enteric-sugar coated tablet formulation

Enteric-sugar coated tablets formulation were prepared at dose of fungal alpha amylase IP (1:1200) 50 mg and pepsin IP (1:3000) 10 mg per tablet. Enteric sugar coated tablet was formulated by preparing core tablet containing fungal alpha amylase, followed by enteric coating, then followed by sugar coating containing pepsin.

Core tablet was prepared by sifting fungal alpha amylase, calcium chloride and lactose through 40 # sieve and mixed for five minutes in cone blender. Binder solution of povidone K-30 in isopropyl alcohol was added to enzyme blend in planetary mixer and kneaded to make a coherent mass. The wet mass was passed through 4 # sieve and the wet granules were dried in oven at room temperature with heaters switched off. The granules were dried till moisture content reaches within 1 -2 %. The dried granules were passed through 16 # sieve. Talc, magnesium stearate and croscarmellose sodium were sifted through 60 # sieve and were added and mixed for five minutes in cone blender. The lubricated granules were compressed into tablets using 7/32" shallow concave punches at an average weight of 65 mg. These tablets were subjected to enteric coating.

Enteric coating solution was prepared by dispersing cellulose acetate phthalate in the mixture of isopropyl alcohol and methylene dichloride and added diethyl phthalate under stirring. Titanium dioxide was passed through 100 # sieve and suspended in the above solution and this coating solution was passed twice through 200 # Sieve. The enteric coating was performed using the conventional coating pan. The cores were prewarmed to about 30°C with the help of hot air blower. The tablets were coated at speed of 20 rpm using spray gun and a fine jet was sprayed at rate of about 20 ml /min with a pressure of 0.5 bar. During spraying, a weak stream of warm air at 40°-45°C was introduced into the bottom part of the pan to keep the cores near room temperature. Spray coating was continued until 8 % increase in coating weight was obtained. The cores were then blown dry with warm air for about 5 minutes at reduced pan rotation. These enteric coated tablets were subjected to sugar coating.

Preparation of sugar coating solutions

Preparation of gum syrup

Gum Acacia was dissolved completely in purified water by heating at 70°C to 75 °C under stirring. Sucrose and sodium benzoate were dissolved completely in purified water and added to sugar syrup and filtered through 200 # nylon cloth.

Preparation of sugar syrup

Sucrose was dissolved in purified water completely by boiling. This solution was filtered through 200 # nylon cloth.

Preparation of dusting powder

Pepsin, talc and calcium carbonate were sifted through 100 # sieve and were mixed in cone blender for 15 minutes.

Preparation of grossing suspension

Grossing Suspension was prepared by mixing talc (100 # size), sugar syrup, gum syrup and titanium dioxide and the solution was mixed uniformly by stirrer.

Preparation of colour coating solution

Colour coating solution was prepared by mixing uniformly titanium dioxide and sugar syrup.

Polishing solution

Carnauba wax and white beeswax were melted into liquid form and were added to ethyl cellulose solution under vigorous stirring to make uniform suspension.

Sugar coating procedure

Enteric coated tablets were loaded into the conventional coating pan and prewarmed at about 30°C. The exhaust was turned on and no air was applied to the tablet bed. The tablets were wet down rapidly with 15 ml of warm gum syrup. The tablets were allowed to roll until they show any signs of becoming tacky, at which time 40 gm of dusting powder was dusted uniformly on the tablets until the tablets roll freely and had no tendency to be tacky. Then tablets were allowed to roll for additionally 15 to 20 minutes to permit the subcoat to set. Five subsequent coats were applied in the same basic manner. The tablets were left in coating pan for about 2 hours without applying air and were jogged periodically. After completion of subcoating, tablets were unloaded form the coating pan and coating pan was cleaned and dried completely.

Subcoated tablets were loaded into the coating pan and prewarmed to about 35°C. The tablets were put in motion and 15 ml of grossing suspension was applied in thin stream to wet the tablets uniformly. Then tablets were permitted to roll continuously with drying air (40° – 45°C) and exhaust on

until dust just begins to appear in the air above the tablets bed. Immediately next coat was applied in similar manner. About four coats were applied. Tablets were dried completely after each coat application. Thereafter 15 ml of six coats of colour coating solution were applied in the similar manner as grossing suspension. Then coating pan was cleaned and again tablets were loaded and exhaust was shut off completely. Thereafter 15 ml of three coats of sugar syrup were applied rapidly without permitting the tablet bed to frost or become dusty. Last sugar syrup coat was applied and the pan shut off while the tablets were still damp. Then the pan was jogged every few minutes for the next 15 minutes. Thereafter tablets were left overnight in the coating pan with close lid without exhaust and hot air. The tablets were transferred to canvas polishing pan. No exhaust or air was used during polishing step. The tablets were put in motion in polishing pan. Thereafter 20 ml of polishing solution were applied to tablets and tablets were allowed to roll until the solvent odour is no longer prevalent. At this point 15 ml of polishing solution was applied immediately and tablets were allowed to roll freely until they begin to shine. Then cold air was applied to remove solvent completely. Tablets were packed in glass bottle and kept for accelerated stability study.

b) Matrix tablet formulation

Matrix tablet formulation was prepared at dose of fungal alpha amylase IP (1:1200) 50 mg and pepsin IP (1:3000) 10 mg per tablet. Fungal alpha amylase, calcium chloride, lactose and cellulose acetate phthalate were sifted through 40 # sieve and mixed for five minutes in cone blender. Binder cellulose acetate phthalate was dissolved in mixture of isopropyl alcohol and methylene chloride completely under stirring and added to enzyme blend and kneaded to make coherent mass in planetary mixer. The wet mass was passed through 4 # sieve and the wet granules were dried in oven at room temperature with heaters switched off. The granules were dried till moisture content reaches within 1 -2 %. The dried granules were passed through 16 # sieve. Talc, magnesium stearate and colloidal silicon dioxide were sifted through 60 # sieve and were added to above dried granules and were mixed for five minutes in cone blender. The lubricated granules were compressed into tablets using 8/32" shallow concave punches at an average weight of 100 mg.

Seal coat solution was prepared by dissolving shellac in isopropyl alcohol. The tablets were loaded into the conventional coating pan and were prewarmed to 30°C. Then Seal coat solution was applied in thin stream onto the tablet core with warm air blowing. Further coatings were given in same basic manner as given for enteric sugar coated tablets, to contain pepsin in sugar coat. Tablets were packed in glass bottle and kept for accelerated stability study.

c) Pellet formulations [14]

Fungal alpha amylase pellets preparation

Sugar syrup was prepared by dissolving sucrose in purified water completely by boiling. This solution was filtered through 200 # nylon cloth and cooled to room temperature. Nonpareil sugar seeds were loaded into conventional coating pan and pan rotation speed was set to about 20 rpm. Nonpareil seeds were prewarmed at 35°C. Fungal alpha amylase, calcium chloride and lactose were sifted through 100 # and mixed uniformly.

The exhaust was turned on and no air was applied to the bed. Thereafter, 10 ml sugar syrup was poured slowly onto the rotating bed of nonpareil seeds, followed immediately by the addition of 35 gm of powder blend. The pellets were allowed to roll freely and had no tendency to be tacky. Then pellets were allowed to roll for additionally 15 minutes to permit the coat to set. The pellets were dried by blowing air at 40°- 45°C onto the rotating bed. Four subsequent coats were applied in the same basic manner. The remaining 5 ml of sugar syrup and talc were sequentially added to the rotating bed. The pellets were rotated for additionally 20 minutes to permit the coat to dry and the excess powder was removed by air. The pellets were dried in oven at 40°C until moisture content was below 2 %. The pellets were sieved and preceded further for enteric coating.

Enteric coating solution prepared by same manner as described in enteric sugar coated tablets. Enteric coating was performed in conventional coating pan with baffle and pan rotation speed was set to about 20 rpm. About 200 gm of pellets of fungal alpha amylase of size 16 # were loaded into coating pan. The pellets were prewarmed to about 30°C with the help of hot air

blower. The pellets were coated using spray gun and a fine jet was sprayed at rate of about 15 ml /min with a pressure of 0.5 bar. During spraying, a weak stream of warm air at 40°- 45°C was introduced into the bottom part of the pan to keep the pellets near room temperature. Spray coating was continued until 8 % increase in coating weight was obtained. The cores were then blown dry with warm air for about 5 minutes at reduced pan rotation.

Pepsin pellets preparation

Pepsin pellets were prepared in similar manner as mentioned for fungal alpha amylase pellets. However these pellets were not enteric coated and were used as it is.

Hard gelatin capsule formulation containing pellets

Hard gelatin capsule formulation was prepared by using enteric coated pellets of fungal alpha amylase at dose of (1:1200) 50 mg and pellets of pepsin at the dose of (1:3000) 10 mg per capsule. Enteric coated pellets of fungal alpha amylase and pellets of pepsin were sifted through 16 # sieve and mixed for 10 minutes. Then hard gelatin capsules were filled at an average weight of 525 mg in clear transparent- clear transparent size "0" hard gelatin capsule by manual capsule filling machine. Capsules were packed in glass bottle and kept for accelerated stability study.

d) Enteric coated powder formulation

Enteric coating of fungal alpha amylase powder was carried out by enteric polymer cellulose acetate phthalate by spray drying method and drug to polymer ratio 1:1, 2:1 were tried. This enteric coated fungal alpha amylase powder was used to prepare capsules, tablets and dry syrup along with pepsin. Enteric coated powder was prepared by dissolving cellulose acetate phthalate in mixed phosphate buffer pH 6.8 with the help of stirrer. Fungal alpha amylase was added to the above solution under stirring and dissolved completely. The solution was filtered through filter paper and proceeded for spray drying.

A laboratory spray-dryer was used for spray drying. The above solution was pumped into the drying chamber at a rate of 1.5 ml/min and pneumatically atomized through a 0.7 mm nozzle using aspiration at 100 % and

atomization air at 1.5 kg/cm^2. The inlet temperature was set at 105° ± 5°C with outlet temperature of 60° ± 5 °C. The resultant powder was blown through the cyclone separator and collected in a container. The Fungal alpha amylase retained after spray drying was estimated. After the spray drying process the product was filled into vials in a moisture free atmosphere.

a) Hard gelatin capsule formulation containing enteric coated amylase and pepsin

Capsule formulation were prepared by using enteric coated particles of fungal alpha amylase at dose of (1:1200) 50 mg and pepsin IP (1:3000) 10 mg per capsule. Capsule formulations were prepared as described in detail in section 4.4.4.1. Capsules were filled at an average weight of 140 mg per in ivory-ivory size "3" hard gelatin capsule by manual capsule filling machine. Capsules were packed in glass bottle and kept for stability.

b) Tablet formulations containing enteric coated amylase and pepsin

Tablet formulation was prepared with enteric coated particles of fungal alpha amylase at dose of (1:1200) 50 mg and pepsin IP (1:3000) 10 mg per tablet. Tablets were prepared as described in detail in section 4.4.5.2a. The lubricated granules were compressed into tablet using 8/32" flat faced beveled edged punches with break line on one side, at an average weight of 100 mg. Tablets were packed in glass bottle and kept for stability.

c) Dry syrup formulation containing enteric coated amylase and pepsin

Dry syrup formulation was prepared with enteric coated fungal alpha amylase powder at dose of (1:1200) 50 mg and pepsin IP (1:3000) 10 mg per 5 ml of reconstituted syrup. Dry syrup was prepared by dry mixing method as described in detail in section 4.4.2.1. Dry syrup 8 gm was filled in 30 ml marking glass bottles and was kept for stability.

4.4.5.3 Formulation of enterosoluble formulations

a) Enteric-sugar coated tablet formulation

The composition of the Batch ES-T-1 is depicted in Table 4.11, 4.12 and 4.13.

Table 4.11 Enteric sugar coated tablet- Batch ES-T-1-tablet core formula

Ingredients	**Qty for 10,000 tablets batch size**
Fungal alpha amylase [1:5555]	162.0 gm
Calcium chloride	20.0 gm
Lactose	430.63 gm
Povidone K -30	22.75 gm
Isopropyl alcohol	115.0 ml
Magnesium stearate	3.25 gm
Talc	4.875 gm
Croscarmellose sodium	6.5 gm
Total weight	650.0 gm
Av weight of tablet	65.0 mg

Table 4.12 Enteric sugar coated tablet - Batch ES-T-1-enteric coating formula

Ingredients	**Qty for 10,000 tablets batch size**
Cellulose acetate phthalate	45.0 gm
Diethyl phthalate	15.4 gm
Methylene dichloride	940 ml
Isopropyl alcohol	100 ml
Titanium dioxide	2.00 gm

Table 4.13 Enteric sugar coated tablet- Batch ES-T-1-sugar coating formula

Ingredients	**Qty for 10,000 tablets batch size**
Gum syrup formula	
Gum acacia	65.0 gm
Sucrose	185.0 gm
Sodium benzoate	5.0 gm
Purified water	260.0 ml
Sugar syrup formula	
Sucrose	185.0 gm
Purified water	95.0 ml
Dusting powder formula	
Talc	330.0 gm
Calcium carbonate	185.0 gm
Pepsin (1:3000)	150.0 gm
Grossing suspension formula	
Talc	40.0 gm
Sugar syrup	55 .0 ml
Gum syrup	30.0 ml
Titanium dioxide	0.4 gm
Colour coating syrup	
Titanium dioxide	1.85 gm
Sugar syrup	110.0 ml
Polishing solution	
Ethyl acetate	35.0 ml
White beeswax	0.3 gm
Carnauba wax	1.2 gm

Evaluation of enteric-sugar coated tablet formulation

1) Evaluation of tablet granules

Following parameters were evaluated as described in section 4.4.2.2

1) Description

Colour and flow of powder were examined

2] Bulk density

3) Tapped density

4] Angle of repose

5) Compressibility index (CI)

6] Moisture content

7] Particle size distribution [15]

All sieves were weighed individually and placed accurately weighed sample on the top (Coarsest) sieve and replaced the lid. Nest of sieves were agitated for 5 minutes. Carefully removed each sieve, reweighed each sieve and determined weight of material on each sieve. Reassembled the nest of sieves and agitated for 5 minutes. Again carefully removed each sieve, reweighed each sieve and determined weight of material on each sieve. This process was repeated until the weight on any of the test sieves did not change by more than 5 % of the previous weight on that sieve. Upon completion of the analysis, the weights of material were reconciled. Total losses must not more than 5 % of the weight of the original sample.

8) Granules to fine ratio [9]

Accurately weighed sample was passed through 60 # sieve and weight percentage of retained and passed sample was determined as granules to fine ratio.

2) Evaluation of the uncoated tablet formulation

Following parameters were evaluated

1) Description

Tablet size, shape, colour, odour were examined.

2) Average weight

Twenty tablets were weighed and average was determined.

3) Uniformity of weight [16]

Twenty tablets were weighed and average weight was determined. Percentage deviation from average weight was determined for individual tablet and compared the results with limits given in Indian Pharmacopoeia as not more than two of the individual weights should deviate from the average weight by more than the percentage deviation and none should deviate by more than twice that percentage. Percentage deviation limit 10 % is given for 80 mg or less than 80 mg average weight of tablet and 7.5 % for more than 80 mg but less than 250 mg average weight of tablet.

4) Diameter

Ten tablets diameter was measured and average was determined.

5) Thickness

Ten tablets thickness was measured and average was determined.

6) Disintegration time [13]

Disintegration test was carried by using disintegration tester and water was used as medium. One tablet was introduced into each tube without and the assembly was suspended into the glass beaker containing water at 37° ± 2°C and operated the apparatus till all tablets were disintegrated. Indian Pharmacopoeia has given the disintegration time limit as 15 minutes for uncoated tablets.

7) Friability [17]

Twenty uncoated tablets were carefully dedusted and accurately weighed and placed in the friability tester. The drum was rotated for 100 times and tablets were removed. The tablets reweighed and percentage loss was determined and limit of friability is not more than 1 %.

8] Hardness

Hardness of the ten uncoated tablets was determined by placing the tablets in the jaws of hardness tester and pressure was applied until the tablets

were broken and recorded the pressure in Kg/cm^2. Average hardness was determined.

9) Moisture content

Moisture content was determined by the method as described in section 4.4.2.2.

10) Assay of fungal alpha amylase

Activity of fungal alpha amylase was determined by Radial diffusion method as described in section 4.3b.

3) Evaluation of the enteric coated tablet formulation

Following parameters were evaluated as described for uncoated tablets

1) Description

2) Average weight

3) Uniformity of weight

4) Diameter

5) Thickness

6) Disintegration time [18]

One enteric coated tablet was placed into each tube of disintegration tester without disc. The assembly was suspended into the beaker containing 0.1N hydrochloric acid at 37° ± 2°C and operated for 120 minutes. Removed the assembly and no tablet should show any signs of cracks. Then the assembly was suspended in beaker containing mixed phosphate buffer pH 6.8, disc was added to each tube and operated the apparatus for 60 minutes. The assembly was removed and test passes if all tablets have disintegrated.

7) Moisture content

8) Drug release [19]

Drug release study was carried out in Indian Pharmacopoeial dissolution apparatus paddle type. One tablet was introduced in each dissolution vessel containing 900 ml of 0.1 N hydrochloric acid at 37° ± 0.5°C and operated at 50 rpm speed for 2 hours. After two hours, these tablets were subjected to buffer stage. Acid was drained from the vessels and added 900 ml of mixed phosphate buffer pH 6.8 and apparatus was operated for 45 minutes. After 45 minutes, an aliquot was withdrawn from each vessel and analyzed for fungal alpha amylase activity. Drug release was taken as average of six determinations.

9) Assay of fungal alpha amylase

4) Evaluation of the sugar coated formulation

Following parameters were evaluated as described for uncoated tablets

1) Description

2) Average weight

3) Uniformity of Weight

4) Diameter

5) Thickness

6) Disintegration time

7) Moisture content

8) Drug release [19]

Drug release study was carried out in dissolution apparatus I (paddle type). One tablet was introduced in each dissolution vessel containing 900 ml of 0.1 N hydrochloric acid at 37° ± 0.5°C and operated at 50 rpm speed for 2 hours. After two hours, an aliquot was withdrawn from each vessel and analyzed for activity of pepsin. Then these tablets were subjected to buffer stage. Acid was drained from the vessels and added 900 ml of mixed phosphate buffer pH 6.8 and apparatus was operated for 45 minutes. After 45 minutes, an aliquot was withdrawn from each vessel and analyzed for fungal alpha amylase activity. Drug release was taken as average of six determinations.

9] Assay of fungal alpha amylase

10] Assay of pepsin

b) Matrix tablet formulation

The composition of the Batches ES-MT-1 to ES-MT-7 is depicted in Table 4.14, 4.15 and 4.16.

Table 4.14 Matrix tablet formulation Batch ES-MT-1 to ES-MT-4 - core tablet formulation

Ingredients	**ES-MT- 1**	**ES-MT- 2**	**ES-MT- 3**	**ES-MT- 4**
Fungal alpha amylase [1:5555]	32.40 gm	32.40 gm	32.40 gm	32.40 gm
Cellulose acetate phthalate	----	2.0 gm	6.0 gm	10.0 gm
Lactose	153.2 gm	157.2 gm	153.2 gm	147.2 gm
Calcium chloride	4.0 gm	4.0 gm	4.0 gm	4.0 gm
Cellulose acetate phthalate (as binder)	2.0 gm	4.0 gm	4.0 gm	6.0 gm
Methylene chloride	90.0 ml	90.0 ml	90.0 ml	90.0 ml
Isopropyl alcohol	20.0 ml	20.0 ml	20.0 ml	20.0 ml
Magnesium stearate	1.4 gm	1.4 gm	1.4 gm	1.4 gm
Talcum	2.0 gm	2.0 gm	2.0 gm	2.0 gm
Colloidal silicon dioxide	1.0 gm	1.0 gm	1.0 gm	1.0 gm
Total weight	200.0 gm	200.0 gm	200.0 gm	200.0 gm
Av weight of Tablet	100.0 mg	100.0 mg	100.0 mg	100.0 mg

Table 4.15 Matrix tablet formulation Batch ES-MT-5 to ES-MT-7- core tablet formulation

Ingredients	**ES-MT- 5**	**ES-MT- 6**	**ES-MT- 7**
Fungal alpha amylase [1:5555]	32.40 gm	32.40 gm	32.40 gm
Cellulose acetate phthalate	14.0 gm	20.0 gm	30.0 gm
Lactose	137.2 gm	129.2 gm	117.2 gm
Calcium chloride	4.0 gm	4.0 gm	4.0 gm
Cellulose acetate phthalate (as binder)	8.0 gm	10.0 gm	12.0 gm
Methylene chloride	90.0 ml	90.0 ml	90.0 ml
Isopropyl alcohol	20.0 ml	20.0 ml	20.0 ml
Magnesium stearate	1.4 gm	1.4 gm	1.4 gm
Talcum	2.0 gm	2.0 gm	2.0 gm
Colloidal silicon dioxide	1.0 gm	1.0 gm	1.0 gm
Total weight	200.0 gm	200.0 gm	200.0 gm
Av weight of Tablet	100.0 mg	100.0 mg	100.0 mg

Table 4.16 Matrix tablet Batch ES-MT-7- sugar coating formula

Ingredients	**Qty for 2,000 tablets batch size**
Seal coat formula	
Shellac	2.0 gm
Isopropyl alcohol	20.0 ml
Gum syrup formula	
Gum acacia	20.0 gm
Sucrose	57.0 gm
Sodium benzoate	1.5 gm
Purified water	80.0 ml
Sugar syrup formula	
Sucrose	57.0 gm
Purified water	30.0 ml
Dusting powder formula	
Talc	100.0 gm
Calcium carbonate	57.0 gm
Pepsin [1:3000]	30.0 gm
Grossing suspension formula	
Talc	12.5 gm
Sugar syrup	17.0 ml
Gum syrup	10.0 ml
Titanium dioxide	0.12 gm
Colour coating syrup	
Titanium dioxide	0.6 gm
Sugar syrup	35.0 ml
Polishing solution	
Ethyl acetate	10.5 ml
White beeswax	0.1 gm
Carnauba wax	0.4 gm

Evaluation of matrix tablet formulations

1) Evaluation of tablet granules

Following parameters were evaluated in similar manner as described in section 4.4.5.3

1) Description

2] Bulk density

3) Tapped density

4] Angle of repose

5) Compressibility index (CI)

6] Moisture content

7] Particle size distribution

8) Granules to fine ratio

2) Evaluation of the tablet formulation

Following parameters were evaluated in similar manner as described in section 4.4.5.3

1) Description

2) Average weight

3) Diameter

4) Thickness

5) Drug release [19]

Drug release study was carried out in Indian Pharmacopoeial dissolution apparatus I (paddle type). One tablet was introduced in each dissolution vessel containing 900 ml of 0.1 N hydrochloric acid at 37° ± 0.5°C and operated at 50 rpm speed for 2 hours. After two hours, these tablets were subjected to buffer stage. Acid was drained from the vessels and added 900 ml of mixed phosphate buffer pH 6.8 and apparatus was operated for four hours. At an interval of one hour, an aliquot was withdrawn from each vessel and analyzed for fungal alpha amylase activity. Drug release was taken as average of six determinations.

6) Friability

7] Hardness

8) Moisture content

9] Assay of fungal alpha amylase

3) Evaluation of the sugar coated formulation

Following parameters were evaluated in similar manner as described in section 4.4.5.3

1) Description

2) Average weight

3) Diameter

4) Thickness

5) Drug release [19]

Drug release study was carried out in Indian Pharmacopoeial dissolution apparatus I (paddle type). One tablet was introduced in each dissolution vessel containing 900 ml of 0.1 N hydrochloric acid at 37° ± 0.5°C and operated at 50 rpm speed for 2 hours. After two hours, an aliquot was withdrawn from each vessel and analyzed for activity of pepsin. Then these tablets were subjected to buffer stage. Acid was drained from the vessels and added 900 ml of mixed phosphate buffer pH 6.8 and apparatus was operated for four hours. At an interval of one hour, an aliquot was withdrawn from each vessel and analyzed for fungal alpha amylase activity. Drug release was taken as average of six determinations.

6) Moisture content

7] Assay of fungal alpha amylase

8] Assay of pepsin

c) Pellet formulation

The composition of the Batches ES-P-1, NES-P-1 and ESP-C-1 is shown in Table 4.17, 4.18 and 4.19 respectively.

Table 4.17 Fungal alpha amylase enteric pellets formulation-Batch ES-P-1

Ingredients	**qty**
Sugar Syrup Preparation	
Sucrose	40.0 gm
Sodium methyl paraben	0.2 gm
Sodium propyl paraben	0.02 gm
Purified water	q.s.to 100 ml
Pellets preparation formula	
Nonpareil sugar seeds [20#]	300.0 gm
Lactose	80.0 gm
Fungal alpha amylase [1:5555]	100.0 gm
Calcium chloride	12.4 gm
Talc	5.0 gm
Sugar syrup	55.0 ml
Enteric coating formula	
Cellulose acetate phthalate	14.0 gm
Diethyl phthalate	4.7 gm
Methylene dichloride	288 ml
Isopropyl alcohol	32 ml
Titanium dioxide	0.6 gm

Table 4.18 Pepsin nonenteric pellets formulation- Batch NES-P-1

Ingredients	**qty**
Nonpareil sugar seeds [20#]	300.0 gm
Lactose	80.0 gm
Pepsin [1:3000]	100.0 gm
Talc	5.0 gm
Sugar syrup	55.0 ml

Table 4.19 Hard gelatin capsule containing enteric pellets of amylase and nonenteric pellets of pepsin- Batch ESP-C-1

Ingredients	**ESP-C-1**
Enteric coated pellets of fungal alpha amylase [1:5555]	133.0 gm
Pellets of pepsin [1:3000]	24.5 gm
Total weight	157.5 gm
Net content per capsule	525.0 mg

Evaluation of pellet formulation

1) Evaluation of the pellets

Following parameters were evaluated as described in section 4.4.5.3

1) Description

2] Bulk density

3) Tapped density

4] Moisture content

5) Compressibility index

6] Particle size distribution

7) Disintegration time

8) Drug release

9) Assay of fungal alpha amylase

10] Assay of pepsin

2) Evaluation of the hard gelatin capsules

Following parameters were evaluated as described in section 4.4.5.3

1) Description

2) Average net weight of capsule

3) Average gross weight of capsule

4) Uniformity of weight

5) Locking length

6) Drug release

7] Moisture content

8] Assay of fungal alpha amylase

9] Assay of pepsin

d) Enteric coated powder formulation

1) Enteric coated powder preparation

The composition of the Batches ECP-1 and ECP–2 is depicted in Table 4.20.

Table 4.20 Enteric coated powder of amylase- Batch ECP-1 and ECP-2

Ingredients	**ECP-1**	**ECP-2**
Fungal alpha amylase [1:5555]	20.0 gm	20.0 gm
Calcium chloride	2.48 gm	2.48 gm
Cellulose acetate phthalate	10.0 gm	20.0 gm
Mixed phosphate buffer pH 6.8 q.s.to	600 ml	600 ml

Evaluation of enteric coated powder

Following parameters were evaluated in similar manner as described in section 4.4.5.3

1) Description

2] Bulk density

3] Moisture content

4) Angle of repose

5] Particle size analysis

The Particle size distribution of the enteric coated powder was measured by Laser light scattering on a Malvern particle size analyzer. Small amount of sample was dispersed in isopropyl alcohol and added to dispersion unit and stirred to reduce the interparticle aggregation and laser obscuration of 15 – 20 % maintained.

6) Scanning electron microscopy (SEM)

Scanning electron microscopy of the enteric coated powder was carried out to know the surface morphology. The enteric coated powder was mounted on double adhesive carbon tape and carefully blown off the excess particles. Morphological evaluation of the particles was conducted using a JEOL Scanning electron microscope with accelerating voltage of 2.5 KV and images were taken at different magnification.

7) Drug release

2) Enteric coated fungal alpha amylase and pepsin hard gelatin capsule formulation

The composition of the Batch ECP-C-1 is given in Table 4.21.

Table 4.21 Enteric coated amylase and pepsin hard gelatin capsule formulation- Batch ECP-C-1

Ingredients	**ECP-C-1**
Enteric coated fungal alpha amylase [1:5555]	12.196 gm
Pepsin [1:3000]	3.0 gm
Lactose	12.524 gm
Talc	0.28 gm
Total weight	28.0 gm
Net content per capsule	140.0 mg

Evaluation of the hard gelatin capsules

I) Evaluation of the hard gelatin capsules blend

Following parameters were evaluated as described in section 4.4.4.2

1) Description

2] Bulk density

4) Tapped density

5) Compressibility index

6) Angle of repose

7] Moisture content

II) Evaluation of the hard gelatin capsules

Following parameters were evaluated as described in section 4.4.4.2

1) Description

2) Average net weight of capsule

3) Average gross weight of capsule

4) Uniformity of weight

5) Locking length

6) Disintegration time

7] Moisture content

8) Drug release

Drug release was determined as described in the section 4.4.5.3

9] Assay of fungal alpha amylase

10] Assay of pepsin

3) Enteric coated fungal alpha amylase and pepsin tablet formulation

The composition of the Batch ECP-T-1 is depicted in Table 4.22.

Table 4.22 Enteric coated amylase and pepsin tablet- Batch ECP-T-1

Ingredients	**ECP-T-1**
Enteric coated fungal alpha amylase [1:5555]	12.196 gm
Pepsin [1:3000]	3.0 gm
Lactose	3.564 gm
Povidone K -30	0.7 gm
Isopropyl alcohol	10.0 ml
Magnesium stearate	0.14 gm
Talc	0.2 gm
Croscarmellose sodium	0.2 gm
Total weight	20.0 gm
Av weight of tablet	100.0 mg

Evaluation of tablet formulation

I) Evaluation of tablet granules

Following parameters were evaluated as described in the section 4.4.5.3

1) Description

2] Bulk density

3) Tapped density

4) Compressibility index

5] Angle of repose

6] Moisture content

7] Particle size distribution

8) Granules to fine ratio

II) Evaluation of the tablet formulation

Following parameters were evaluated as described in the section 4.4.5.3

1) Description

2) Average weight

3) Uniformity of weight

4) Diameter

5) Thickness

6) Disintegration time

7) Friability

8] Hardness

9) Moisture content

10) Drug release

11] Assay of fungal alpha amylase

12) Assay of pepsin

4) Enteric coated fungal alpha amylase and pepsin dry syrup formulation

The composition of the Batch ECP-DS-1 is depicted in Table 4.23.

Table 4.23 Enteric coated amylase and pepsin dry syrup formulation-Batch ECP-DS-1

Ingredients	**ECP-DS-1**
Enteric coated fungal alpha amylase	7.320 gm
Pepsin [1:3000]	1.8 gm
Sucrose	146.09 gm
Sodium benzoate	0.24 gm
Quinoline yellow colour	0.05 gm
Guar gum	1.0 gm
Colloidal silicon dioxide	0.5 gm
Pineapple powder flavour	3.0 gm
Total weight	160.0 gm

Evaluation of the dry syrup formulations

I) Evaluation of the dry syrup formulations

Following parameters were evaluated as described in section 4.4.2.2

1) Description

2) Bulk density

3) Tapped density

4) Compressibility index

5) Angle of repose

6] Moisture content

7) Drug release

8] Assay of fungal alpha amylase

9] Assay of pepsin

II) Evaluation of the reconstituted dry syrup formulations

Following parameters were evaluated as described in section 4.4.2.2

1) Description

2) pH

3) Specific gravity

4] Assay of fungal alpha amylase

5] Assay of pepsin

4.4.6 INCLUSION COMPLEX

A) EXPERIMENTAL SECTION

4.4.6.1 Preparation of inclusion complex

Inclusion complex of fungal alpha amylase and beta-cyclodextrin were prepared [20,21] by adding fungal alpha amylase to 50 mM Tris buffer at desired pH and stirred to dissolve completely. To this solution beta-cyclodextrin was added and solution was stirred slowly at desired temperature for desired interval of time. After stirring, fungal alpha amylase activity was estimated.

4.4.6.2 Preformulation study

a) Factors influencing inclusion complex formation

1) Enzyme concentration

Inclusion complex of fungal alpha amylase in different concentration such as 9.8, 49, 98 and 196 μM were carried out with beta-cyclodextrin at molecular ratio 3:1 and stirred for 2 hours at 37 °C in buffer (50 mM Tris, pH 8.5) . Recovery of fungal alpha amylase was estimated.

2) Stirring time

Inclusion complex of fungal alpha amylase at 9.8 μM concentration was carried out with beta-cyclodextrin at molecular ratio 3:1 and stirred for 2 hours, 4 hours, 6 hours, 8 hours and 10 hours at 37 °C in buffer (50 mM Tris, pH 8.5). Recovery of fungal alpha amylase was estimated.

3) pH and temperature

Fungal alpha amylase inclusion complex was carried out with beta-cyclodextrin at molecular ratio 3:1 and at 9.8 μM concentration for 2 hours. This, inclusion complex was carried out at pH from 5.0 - 9.0 and temperature 15° to 50 °C and recovery of fungal alpha amylase was estimated.

4) Ionic strength

Fungal alpha amylase inclusion complex was carried out with beta-cyclodextrin at molecular ratio 3:1 in 50 mM Tris buffer, pH 8.5 with addition of sodium chloride 5 mM to 500 mM concentration, calcium chloride 0.6 mM to 4.5 mM concentration separately and solution was stirred for 2 hours at 37°C. The activity of fungal alpha amylase enzyme was estimated.

b) Aggregation inhibition study

100 ml of 8 M Urea solution was prepared in 0.1 M hydrochloric acid and to this solution 50 mg (9.8μM)of fungal alpha amylase added. This solution was kept at room temperature for 18 hours. Thereafter 20 ml of this denatured fungal alpha amylase in urea solution was rapidly diluted with 20 ml of 50 mM Tris buffer at pH 8.5 and at every minute, aggregation was monitored by light scattering at 400 nm up to 20 minutes.

Similarly, 20 ml of denatured fungal alpha amylase in urea solution was rapidly diluted with 20 ml of 50 mM Tris buffer solution having pH 8.5 containing 20 mg of beta-cyclodextrin. At every minute, aggregation was monitored by light scattering at 400 nm up to 20 minutes.

c) Recovery of activity in excess of initial activity

Inclusion complex of fungal alpha amylase and beta-cyclodextrin were carried out at 1:1 Host: Guest [beta-cyclodextrin: fungal alpha amylase] molecular ratio as per following given method. 50 mg of fungal alpha amylase was added to 100 ml of 50 mM Tris buffer having pH 8.5, stirred to dissolve completely. To this solution 1.12 mg of beta-cyclodextrin was added and this solution was stirred slowly at 37°C for 2 hours. After 2 hours of stirring, fungal alpha amylase activity was estimated. Similarly, inclusion complex of fungal alpha amylase and beta-cyclodextrin were carried out at different molecular ratio such 2:1, 3:1, 4:1, 5:1,6:1, 7:1, 8:1, 9:1 & 10:1. The amount of fungal alpha amylase was determined. The fungal alpha amylase concentration kept same for all ratios (9.8 μM).

d) Characterization of inclusion complex

Inclusion complex of fungal alpha amylase at 9.8 μM concentration was carried out with beta-cyclodextrin at molecular ratio 3:1 and stirred for 2 hours at 37 °C in buffer (50 mM Tris, pH 8.5).

Following analysis were carried out for confirmation of inclusion complex between fungal alpha amylase and beta-cyclodextrin in Tris buffer.

UV spectroscopy studies

UV spectral measurements were carried out using UV Spectrometer in absorbance range between 190 and 400 nm.

Fluorescence spectroscopy studies

Fluorescence spectral measurements were performed using Spectrofluorophotometer. Fluorescence spectra of the above solution were taken at 425 nm excitation wavelength and emission wavelength in the range 200- 500 nm.

FTIR spectroscopy

FTIR spectral measurements were performed using FTIR Spectrometer. The above solution was placed in liquid cell and spectra were taken in the range 4000- 400 cm^{-1} and the buffer contribution was subtracted.

H1-NMR spectroscopy

H1-NMR spectral measurements were performed using a NMR spectrometer. The above solution was vacuum dried to remove moisture and then diluted in D_2O solvent and subjected to NMR spectroscopy.

Differential scanning calorimetry (DSC)

Measurements of differential scanning calorimetry were obtained on a Mettler TA – 4000 thermoanalyser system. The sample solution were introduced into the DSC oven and then heated at the rate of 10°C per minute up to 200°C and thermograph recorded.

4.4.6.3 Preparation of oral liquid formulation with inclusion complex

Inclusion complex was prepared at 3:1 molecular ratio and then this complex was incorporated in oral liquid formulation at dose of fungal alpha amylase IP (1:1200) 50 mg and pepsin IP (1:3000) 10 mg per 5 ml at different pH and with different syrup base. The composition of the batches IC-L-1 to IC-L-4 is shown in Table 4.24 and 4.25. Oral liquid formulation prepared in similar manner as described in section 4.4.1.2. These formulations were subjected to accelerated stability study at 45°C, 30°C, and refrigerated condition (2°- 8° C). The activity of fungal alpha amylase and pepsin was estimated at an interval of 0,1,2,3 months.

Table 4.24 Oral liquid formulation with inclusion complex Batch IC-L-1 and IC-L-2

Ingredients	**IC-L-1**	**IC-L-2**
Fungal alpha amylase [1:5555]	1.080 gm	1.080 gm
Pepsin [1:3000]	1.500 gm	1.500 gm
Beta-cyclodextrin	0.073 gm	0.073 gm
Sucrose	275.0 gm	275.0 gm
Sodium methyl paraben	1.0 gm	1.0 gm
Sodium propyl paraben	0.1 gm	0.1 gm
Bronopol	0.5 gm	0.5 gm
Caramel colour	0.025 gm	0.025 gm
Cardamom essence	0.5 gm	0.5 gm
Tris buffer 50 mM q.s.to	500.0 ml	500.0ml
pH adjusted to	7.0	8.5

Table 4.25 Oral liquid formulation with inclusion complex Batch IC-L-3 and IC-L-4

Ingredients	**IC-L-3**	**IC-L-4**
Fungal alpha amylase	1.080 gm	1.080 gm
Pepsin	1.500 gm	1.500 gm
Beta-cyclodextrin	0.073 gm	0.073 gm
Sorbitol powder	275.0 gm	275.0 gm
Sodium methyl paraben	1.0 gm	1.0 gm
Sodium propyl paraben	0.1 gm	0.1 gm
Bronopol	0.5 gm	0.5 gm
Caramel colour	0.025 gm	0.025 gm
Cardamom essence	0.5 gm	0.5 gm
Tris buffer 50 mM q.s.to	500.0 ml	500.0ml
pH adjusted to	7.0	8.5

4.4.6.4 Evaluation of the oral liquid formulations

Following parameters were evaluated as described in section 4.4.1.3

1) Description

2) pH

3) Specific gravity

4] Assay of fungal alpha amylase

5] Assay of pepsin

B) COMPUTER AIDED MOLECULAR MODELING

1) Preparing the enzyme model

The starting structure for simulations was taken from the 2.10 A° resolution refined x-ray crystal structure of fungal alpha amylase from source *Aspergillus oryzae* (Brookhaven Protein Data Bank, PDB code 6taa)[22]. All the hydrogens were added to the enzyme structure. X-ray crystallographic water molecules associated with enzyme were removed. Structure of beta-cyclodextrin were taken from Brookhaven Protein Data Bank, PDB code GLC [22].

2) Defining the binding pocket

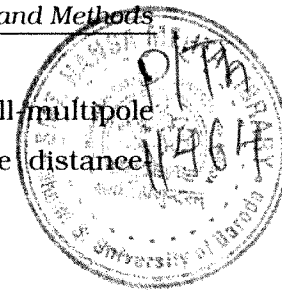
The binding pocket residues were defined using Catalytic Site Atlas (CSA) version 2.0.10 entry for 6taa and six residues were collected to make up the binding site subset [23]. These residues also confirmed by 'castP' pocket information for 6taa molecule [24].

3) Docking procedure

All computations were performed on a Silicon Graphics ONYX 300 Base workstation with Infinite Reality 3 Graphics, using the Insight II (v. 2000.1) software package from Molecular Simulation Inc. (MSI), San Diego, CA. The Insight II program discover 3 (v.98) was used for all molecular mechanics and molecular dynamics calculations, employing the second generation cff91 force field [25-27].

The ligand was reoriented in the defined binding pocket for the start of the affinity calculations to avoid bias towards the crystal structure position. The assembly was subjected to a 1000 iterations Monte Carlo (MC) search with number of structure to generate 50 and to accept 50. The nonbonded interactions were calculated by cell-multipole method [28] and the distance-dependent dielectric constant was set to 4 and energy range of 200 and tolerance of 1e+06 and 1000 steps minimization was set.

The low energy structures of docking simulations were subjected to Molecular dynamics (MD) simulation. The MD calculations were done using



Velocity Verlet algorithm [29] at constant volume with the cell-multipole method for the calculation of nonbonded interactions and the distance-dependent dielectric constant was set to 4.

The initial and final temperatures of the simulated annealing step were set to 500 and 300 K respectively with time steps of 1.0 fs. The system was brought to 300 K over 50 ps and equilibrated over 10000 steps. RMS deviations of beta-cyclodextrin- fungal alpha amylase complex model from fungal alpha amylase X-ray crystal structure, geometry were calculated. Thereafter Ligand Protein Contacts (LPC) analyses were performed using LPC Software [30].

The value of the accessible surface area for both fungal alpha amylase and inclusion complex were calculated using MOLMOL version 2K.2 software [31].

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