Chapter 6

SUMMARY & CONCLUSION

6. SUMMARY AND CONCLUSIONS

In Indian market, alpha amylase with pepsin formulations is available only in syrup and drops form. These formulations are not only having shorter shelf life, are also unstable at room temperature through out their shelf life. The objective of this study was to develop some conventional stable fungal alpha amylase formulations and also to develop some novel formulations for better stability as well as efficacy.

Effect of pH, heat and salts on fungal alpha amylase activity was studied. It was found that fungal alpha amylase as raw material was stable at pH 6-9 and shown better stability in solid state at 2-8°C up to 27 months and at 30°C up to 13 months but at 45°C and 40°C/75 % RH, it shown considerable degradation. Stability of fungal alpha amylase in liquid state at 2-8°C was found to be up to 6 months, at 30°C up to 3 months, and at 45°C enzyme was totally degraded in 3 months. Calcium chloride was found to increase the stability of fungal alpha amylase than magnesium chloride and sodium chloride.

When fungal alpha amylase was treated with chelating agent like disodium EDTA, its activity was found to be lost. Based on this experiment, it was concluded that the calcium ion is essential for fungal alpha amylase activity and stability of enzyme. In presence of calcium ions, the stability of fungal alpha amylase was found to be increased. The interaction between fungal alpha amylase and cofactor calcium ion was studied by ultraviolet, fluorescence and infrared spectroscopy and no alterations were found in structure of fungal alpha amylase after interacting with Calcium Chloride.

Fungal alpha amylase formulations such as oral liquid syrup, dry syrup, oral drops, and hard gelatin capsules were formulated and stabilized by addition of cofactor calcium chloride. All these formulations were characterized by physical and chemical means. From accelerated stability study, shelf life of oral liquid syrup was estimated to be 21.7 months, dry syrup 26 months, oral drops 20 months, and hard gelatin capsule 24.4 months. Dry syrup was found to be most stable among these formulations.

Fungal alpha amylase in-vitro stability in gut pH was carried out and it was observed that enzyme was not stable in acidic medium. Also market samples in-vitro stability in gut pH confirmed that fungal alpha amylase could not remain active in acidic medium. It is concluded that to get full activity of fungal alpha amylase, it is essential to protect enzyme from acidic environment of stomach by developing acid resistant enterosoluble formulations.

Enterosoluble formulations such as enteric sugar coated tablets, sugar coated matrix tablets and hard gelatin capsules containing enteric coated pellets were developed. Enteric sugar coated tablet was formulated by preparing core tablet containing fungal alpha amylase along with cofactor, followed by enteric coating, and then sugar coated using sugar coating solution containing pepsin. Shelf life was estimated from accelerated stability study to be up to 26 months. Sugar coated matrix tablets were developed by incorporating fungal alpha amylase in the matrix of cellulose acetate phthalate along with cofactor and pepsin was incorporated in sugar coat for immediate release. Similarly hard gelatin capsules were prepared containing mixture of enteric coated pellets of fungal alpha amylase along with cofactor and uncoated pellets of pepsin. Shelf life estimated to be 24 months for sugar coated matrix tablets and 32 months for hard gelatin capsule containing enteric coated pellets of amylase.

Enteric coated fungal alpha amylase powder prepared by spray drying method using enteric polymer such as cellulose acetate phthalate along with cofactor and drug-polymer ratio 1:1 shown good protection against acid environment. Spray dried powder was characterized for particle size distribution, scanning electron microscopy and flow property. Using this spray dried powder, hard gelatin capsules, Tablets, dry syrup were formulated and found to be stable at acid medium. Shelf life estimated to be 28.4 months for hard gelatin capsules, 29.6 months for tablet and 30 months for dry syrup.

Dissolution study of all the developed enterosoluble formulations showed that pepsin was released in 0.1 N hydrochloric acid medium and fungal amylase

resisted release in acidic medium but released slowly in mixed phosphate buffer pH 6.8, which confirmed the full protection of alpha amylase from acidic pH.

Inclusion complex of fungal alpha amylase with beta-cyclodextrin was tried for stabilization of fungal alpha amylase. It was found that 3:1 Host: Guest molecular ratio recovered 185 % fungal alpha amylase activity over the initial activity. Factors affecting inclusion complex were studied like aggregation inhibition, effect of enzyme concentration, effect of stirring time, effect of pH and temperature, effect of ionic strength upon inclusion complex. The complex was characterized and confirmed by Ultraviolet spectroscopy, Fluorescence spectroscopy, Infrared spectroscopy, proton-Nuclear Magnetic Resonance Spectroscopy and Differential Scanning Calorimetry. Using this complex of fugal alpha amylase with beta-cyclodextrin, oral liquid formulation were prepared and found to be stable up to 26 months.

Molecular modeling studies were carried out to know the interaction between beta-cyclodextrin and amino acid residues of fungal alpha amylase. The molecular modeling study was performed using INSIGHT II version 2000.1 running on a Silicon Graphics ONYX 300 Base workstation with InfiniteReality3 Graphics. Advanced Class II force field (CFF91) implemented in Discover 3 program (Version 98) was used for all molecular mechanics calculations. Fungal Alpha amylase and beta-cyclodextrin structure were downloaded form Protein Data Bank (PDB). Beta-cyclodextrin was docked into the active site pocket of alpha amylase using Monte Carlo minimization method. This gave lowest energy minimized structures. Then these lowest energy minimized structures subjected to molecular dynamics simulations at 500 K to 300 K temperatures. From RMS deviations of inclusion complex model from alpha amylase X-ray crystal structure, geometry was calculated. Then accessible surface area, Ramchandran plots, Ligand Protein Contacts (LPC) analyses were performed using Software. Molecular modeling study resulted in noncovalent interaction like hydrogen bonding between beta-cyclodextrin and residue of active site of fungal alpha amylase.

Based on the above, it is concluded that fungal alpha amylase is very sensitive to pH and heat. If liquid formulations maintained at pH 6-9 and kept at 2-8°C, longer shelf life can be achieved. But ideally maintaining 2-8 °C is not possible through out the market, so these formulations need to be stabilized further. Following three approaches were found to give stabilized formulation:

1) **Addition of cofactor:** Addition of Calcium cofactor which is essential for its catalytic activity found to stabilize many formulations containing enzymes and this method is economically effective also.

2) **Preparing enterosoluble formulations along with cofactor:** Since stability study of existing Indian fungal alpha amylase formulations in gut, showed major degradation before reaching to intestine. Making enterosoluble alpha amylase formulations showed promising effective targeted drug delivery system.

3) **Preparing inclusion complex with beta-cyclodextrin:** This present inclusion complex method for enhancement of activity and stability of enzyme comprising contacting enzyme in aqueous medium with beta-cyclodextrin, found to be effective to increase the activity and stability of enzyme more than 100% of its initial enzymatic activity and there by reducing the quantity and cost of enzyme.

Finally, it is concluded that the acid resistant enterosoluble formulations along with cofactor were found to be stable as well as effective drug delivery system for fungal alpha amylase.