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Immobilization of Digestive Enzymes for Improvisation of Shelf life in Their Formulations

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SUMMARY & CONCLUSIONS

Digestive enzymes are considered beneficial for dyspepsia and as appetite stimulants. Marketed preparation contains α -amylase, papain, and pepsin, etc. either alone or with vitamins, or with carminatives are available for use. Number of marketed formulations containing these enzymes and other vitamins were analyzed and it was found that the concentration of the enzymes were less than the labeled claim (might be because of incompatibility of enzymes with vitamins and carminatives). Further, pharmaceutical formulations containing digestive enzymes need to be stored at cold (2 – 8°C) or cool (8 – 25 °C) temperature conditions and still has the self-life of not more than one year.

Entrapment of the enzyme in biodegradable biopolymers like chitosan, alginate and carrageenan using the technique of the ionotropic gelation or polyelectrolyte complex (PEC) may improve the stability of the parent enzymes and make it less prone to interference of various formulation excipients. Immobilized enzymes are stable at higher temperature and might be stored at room temperature with extended shelf-life. Above advantages are of great commercial interest for the pharmaceutical industries hence it was the objective of the research to develop an extended shelf-life formulations of various digestive enzymes by entrapment in biodegradable biopolymers which results in better and efficient utilization of enzyme.

For the study purpose the three digestive enzymes namely, α -amylase, papain, and pepsin were selected. Similarly, three biodegradable biopolymers namely, alginate, κ -carrageenan, and chitosan were selected for the study. For the sake of simplicity the

work of the thesis has been divided into four sections: (I) Introduction, (II) Materials and Methods, (III) Results and Discussions, and (IV) Summary and Conclusions.

Chapter 1 defines enzyme (holoenzyme), apoenzyme, coenzyme and enzyme immobilization as well. Further it classified the immobilization methods broadly into: (i) carrier-binding, (ii) cross-linking, and (iii) entrapment with enumeration of the advantages of enzyme immobilization. A detailed look of enzyme immobilization method with sub-classification is also depicted in the same chapter. Neural network (NN) has been successfully applied to many pharmaceutical areas in recent years e.g.: QSAR analysis and drug modeling, pharmacokinetic–pharmacodynamic studies, optimization and pharmaceutical formulation development, powder flow, compound determination using HPLC, analysis of NMR spectra, prediction of drug release profile, prediction of physicochemical properties, prediction of octanol-water partition coefficient, prediction of solubility, etc. Further, NN models might generalize better than regression models since regression analyses are dependent on predetermined statistical significance levels (i.e. less significant terms are not included in the model). With the NN method all data are used potentially making the models more accurate. Hence was used in the present study to predict the response surface. Chapter 1 provides brief description on the very essential parts of NN and its functions along with application in the present study.

A concise yet informative details about the enzymes used in the study were given in the Chapter 2. Enzyme nomenclature with sub-classes as per the recommendations of the Nomenclature Committee (NC) of the International Union of Biochemistry and Molecular Biology (IUBMB) is given in this chapter. Relative positions of the enzymes studied in the classification tree were shown in chart form. General information, chemical

structures, 3D structures, amino acids at the active site, their roles, catalytic mechanism, etc have also been described. The received enzyme samples were characterized by means of FTIR and DSC and confirmed against the pharmacopoeial values or other reported values. At the end of each enzyme profile, different reported methods for estimation of enzymes have been enumerated.

Physico-chemical properties of three selected biodegradable biopolymers, namely alginate, carrageenan, and chitosan, have been listed in the Chapter 3. The information were given under the subheadings of general information, source of materials, chemical structure, composition of repeating unit(s), gelling mechanism, biocompatibility of biopolymers, applications, etc. Finally, the received samples were characterized by FTIR and DSC. Alginate sample contains guluronic (G) and mannuronic (M) acid repeating units. Consecutive GG block constitute an 'egg-box' like structure where in some divalent cations can interact and form a hard gel. This is called as ionotropic gelation of alginate. The affinity of cations to alginates is in the following order: $\text{Pb} > \text{Cu} > \text{Cd} > \text{Ba} > \text{Sr} > \text{Ca} > \text{Co, Ni, Zn} > \text{Mn}$. The structure of kappa and iota carrageenan allows segments of the two molecules to form so called double helices which bind the chain molecules (line K^+ in this case) in the three dimensional network, a gel. Lambda carrageenan has a structure that does not allow such double helix formation and thus does not hard in presence of any metal ions. Kappa and iota carrageenan can form gel with Li^+ , Na^+ , K^+ , Rb^+ or Cs^+ . Chitosan is cationic polymer and can form polyelectrolyte complex with many oppositely charged biopolymers. Conventionally the chitosan-alginate polyelectrolyte complexes have been studied by dropping the alginate solution containing the drug / enzyme to the chitosan solution containing the hardening salt (CaCl_2). It was reported that the reversed chitosan-alginate prepared by dropping chitosan solution to the

alginate solution does not have enough hardness even after hardening up to 3 hr. However, in the present study we prepared the reversed chitosan-alginate beads with desired hardness with less hardening time by reacting chitosan-alginate at the pH values where both polymers were in fully ionized form, and thus result in very dense cross-linked polyelectrolyte complex (PEC). Several diagrams have been presented simulating the reaction between chitosan and alginate at the reaction conditions.

Literature survey is accommodated in the Chapter 4. Literature on alginate beads, carrageenan beads and chitosan-alginate PEC have been reviewed in this section.

Chapter 5 describes the scope of the work. The problem associated with the present system and a remedial action with possible advantages have been mentioned. As has been depicted above, the pharmaceutical formulations containing digestive enzymes need to be stored at cold (2 – 8°C) or cool (8 – 25 °C) temperature conditions and still has the self-life of not more than one year. Immobilized enzymes are stable at higher temperature and might be stored at room temperature with extended shelf-life. Above advantages are of great commercial interest for the pharmaceutical industries hence it was the objective of the research to develop an extended shelf-life formulations of various digestive enzymes by entrapment in biodegradable biopolymers.

Chapter 6 belongs to section II of the thesis, where in the methods and materials of preparing alginate beads were explained. The alginate beads were prepared by dropping the enzyme containing alginate solution to the calcium chloride solution. Three main parameters were studied: (i) alginate concentration, (ii) calcium chloride concentration, and hardening time. This system was studied for α -amylase and papain solution. The

hardened beads were filtered off using Büchner funnel and dried. Estimation of α -amylase from the beads was carried out by modified Smith and Roe method. Starch was used as a substrate here. The mixture was incubated for 3 minutes at 25° and then reaction was stopped with 1 N HCl. Finally, it was diluted and 0.01 N iodine solution was added followed by measuring the absorbance at 660 nm. Similarly, papain was estimated by modified casein digestion method of USP XXVI in presence of cysteine hydrochloride. Different aliquots of standard papain solution were added to buffered substrate (hammersten-type casein, pH 6.0 \pm 0.1) and incubated for 60 min at 40°C. Digestion process of casein was stopped by adding 30% w/v trichloroacetic acid solution and allowed to stand for 30 – 40 min at 40°C. Digested amino acids were filtered and absorbance was measured at 280 nm against their respective blanks. The method was found to be linear over an analytical range of 3 – 100 μ g/ml with correlation coefficient (r) of 0.9996. Limit of detection, limit of quantitation, and regression equation were found to be 0.77 μ g/ml, 2.57 μ g/ml, and $y = 0.0042x - 0.0033$ respectively. Method of determining various parameters like particle size, angle of repose, neural network topology, FTIR, DSC, SEM etc have been described.

Materials and methods regarding the carrageenan beads have been described in Chapter 7. The carrageenan beads were prepared by dropping enzyme containing κ -carrageenan solution to the potassium chloride solution. Various process parameters, like carrageenan concentration, potassium chloride concentration, and hardening time were studied for effect on dependant variables. This system was studied for all three digestive enzymes (i.e. α -amylase, papain, and pepsin). α -Amylase and papain were estimated by the method given above. The activity of pepsin was measured by the modified method of Anson wherein the quantity of peptides (from hemoglobin), non-precipitable by TCA was

determined and assayed using the phosphomolybdotungstic reagent. The absorbance of the resultant solution was measured at 540 nm at room temperature. Method of determining various parameters like particle size, angle of repose, FTIR, DSC, SEM, effect of pH on release profile, Kopcha parameters, dissolution mechanism, etc have been described.

Chapter 8 explained the materials and methods for the preparation of chitosan-alginate polyelectrolyte complex (PEC) gel. In contrast to the conventional method, the chitosan-alginate PEC, in the present study, was prepared by reversed method i.e. chitosan solution was added drop wise to the alginate solution. Here also, three process parameters viz, chitosan concentration, alginate concentration, and hardening time, were varied to see the effect of them on the independent parameters. This study was studied for α -amylase and papain, and not studied for pepsin because, it contains chitosanase enzyme, which digests the chitosan. Procedure for determining various parameters like particle size, angle of repose, FTIR, DSC, SEM, effect of pH on release profile, Kopcha parameters, dissolution mechanism, etc have been described.

Chapter 9 depicts the results and discussion for the alginate beads. The experiments of the alginate-amylase beads were carried out by Doehlert Uniform Shell design. The optimization of the alginate-amylase beads using the desirability function resulted in more than 85% entrapment and less than 15 min of T_{50} at low levels of all three process variables (1.3% sodium alginate, 0.0856 M calcium chloride, and 20.9 min hardening time). The enzyme release data show a good fit to the first-order release kinetics. The Kopcha model monitored the diffusion to erosion ratio A/B . Initially A/B was <1 however, diffusion term A increased in the course of time as diffusion appeared. This explained the

biphasic nature (erosional first and diffusional latter) of the '*in vitro*' release profile. Calcium alginate beads have non swelling property in acidic environment while swell and disintegrate in intestinal fluid. Hence, there was very low amount of α -amylase released in the acidic media (pH 1.2, 4.0, and 5.4). The swelling and disintegration of alginate beads in intestinal fluid (pH 6.8) were due to the affinity of calcium to phosphate and sodium/calcium exchange. The shelf-life of the entrapped enzyme has been increased to 3.39 years from 0.99 years of the conventional dosage form.

Alginate-papain beads were studied by 3^3 full factorial study. The optimization was carried out by overall desirability function and neural network. However, then also no significant difference observed between the results of both the cases. It was found that the drug entrapment capacity of papain was significantly enhanced by decreasing calcium chloride concentration and hardening time as well as by increasing in sodium alginate concentration. Drug entrapment of papain was confirmed using FTIR and DSC. The shelf-life of the entrapped enzyme was found to be increased to 3.6 years compared to 1.01 year of the marketed formulation.

Chapter 10 includes the information regarding the ionotropically corsslinked κ -carrageenan beads. Carrageenan-amylase beads were prepared by 3^3 full factorial design. The optimization of the process using the composite index resulted in more than 73% entrapment and more than 74 min of T_{90} at high levels of all three process variables. T_{50} and T_{90} were increased with increase in all three process variables. Percentage entrapment and particle size were found to be directly proportional to κ -carrageenan concentration and inversely proportional to potassium chloride concentration and hardening time. Mathematical analysis of the different drug release modalities has evidenced that enzyme release from carrageenan beads follows Korsmeyer-Peppas' power law equation with

super case-II transport mechanism. Furthermore, investigation of release profile by Kopcha model revealed that enzyme release is due to erosion and not by diffusion. In addition, the surface roughness decreased with increase in κ -carrageenan concentration. Shelf-life of the immobilized enzyme formulation was found to be 3.53 years compared to 0.99 years of the marketed formulation.

Ionotropically cross-linked κ -carrageenan-papain beads are a promising method for improving the stability of entrapped papain and can find a place in the design of multiparticulate drug delivery systems. The optimization of the process using the Doehlert design and composite index resulted in more than 82% entrapment and more than 55 min of T_{90} at optimized process variables. T_{50} and T_{90} were increased with increase in all three process variables. Percentage entrapment and particle size were found to be directly proportional to κ -carrageenan concentration and inversely proportional to potassium chloride concentration and hardening time. Texture analysis indicated a direct proportionality between degree of cross-linking and potassium chloride concentration, and an inverse relationship between surface roughness with increase in κ -carrageenan concentration. The shelf-life of the immobilized enzyme was found to be 3.63 years compared to 1.01 year of the marketed formulation.

The carrageenan-pepsin beads were studied using Box-Behnken design and response surface methodology. The optimization of the process using the response surfaces resulted in more than 81% entrapment and less than 36 min of T_{90} at optimized process variables. T_{50} and T_{90} were increased with increase in all three process variables. Percentage entrapment and particle size were found to be directly proportional to κ -carrageenan concentration and inversely proportional to potassium chloride concentration and hardening time. Further, the investigation of release profile by Kopcha model revealed that enzyme release up to 15 min was due to diffusion followed by erosion.

Stability of the entrapped formulation can found to be increased up to 3.24 years which was 0.97 in the case of the marketed formulation.

Chapter 11 depicts the results of chitosan-alginate beads. Reversed chitosan-alginate α -amylase PEC beads were studied by 33 full factorial design and response surface design. The optimization of the process using the response surfaces resulted in $> 90\%$ entrapment and > 48 min of T_{90} . T_{50} and T_{90} were increased with increase in alginate concentration and hardening time while decreased with increase in chitosan concentration. Percentage entrapment was found to be directly proportional to chitosan and alginate concentration while inversely proportional to the hardening time. Mathematical analysis of the different drug release modalities and Kopcha model revealed that after 35 min the enzyme was released due to the burst effect. FTIR and DSC study confirmed the entrapment of α -amylase in the chitosan-alginate beads. Texture analysis demonstrated the mesh-like fine structure due to the PEC reaction in absence of added salt. Accelerated and long term stability study illustrated considerably improved shelf-life of α -amylase entrapped in chitosan-alginate beads (3.68 years) than the conventional dosage form (0.99 year).

Reversed chitosan-alginate papain PEC beads exhibited promising stability improvement of entrapped papain (3.75 years) compared to 1.01 years of the marketed formulation. The optimization of the process using central composite design and the response surfaces resulted in $> 90\%$ entrapment and > 53 min of T_{90} . T_{50} and T_{90} were increased with increase in alginate concentration and hardening time while decreased with increase in chitosan concentration. Percentage entrapment was found to be directly proportional to chitosan and alginate concentration. Mathematical analysis of the different drug release modalities and Kopcha model revealed that after 40 min the enzyme was released due to the burst effect. FTIR and DSC study confirmed the entrapment of papain in the chitosan-

alginate beads. Texture analysis demonstrated the mesh-like fine structure due to the PEC reaction in absence of added salt. Reversed chitosan-alginate PEC can be employed for number of application due to short time of reaction, simplicity and reactivity in salt-free condition

In conclusion, the results of this thesis demonstrate that the enzyme immobilization is the effective tool for improving the shelf-life significantly. In all the case the shelf-life was significantly improved. The reversed chitosan-alginate PEC were reported to be fragile even after 3 hr stirring. However, the proper pH selection of both the polymers keep them in the ionized form and increase the cross-linking density. Further, at the appropriate pH conditions, the enzymatic degradation of chitosan can be prevented. The thesis also emphasis on to the mathematical modeling of 'in vitro' dissolution study. With the help of suitable release profile, the drug / enzyme release mechanism can be predicted. Based on the provided methodologies and depending on the site of action for enzyme within the gastrointestinal tract, desirable beads can be easily and reproducibly manufactured. Results of present study seem to be of value for the pharmaceutical industries associated with digestive enzymes formulations, for those pharmaceutical scientists who are engaged in site-specific delivery, as well as the oral delivery of enzymes, peptides, proteins and ulcerogenic agents and/or anti-inflammatory agents.