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PREPARATION AND EVALUATION OF INCLUSION COMPLEXES OF PAPAIN WITH HP- β-CD

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ABSTRACT

Inclusion complexes of β-cyclodextrin are useful to improve solubility and stability of drugs and therapeutic proteins. Protein molecule were actively incorporated in to HP- β-CD for improving the dissolution and bioavailability. Studies permit to understand their properties and possible therapeutic applications. Papain (digestive enzyme) was chosen and the factors, namely substrate concentration, pH of the medium, and temperature influencing enzyme activity were investigated. A suitable analytical method was established for papain at λ_{max} 660 nm. It was verified that there is no interference of hydroxy propyl β-cyclodextrin on the analytical wavelength of enzyme. Enzymatic activity of papain was assayed at increasing concentrations of hydroxy propyl β-cyclodextrin. The enzyme activity was decreased as the concentration of HP-β-CD increased, indicating inaccessibility of papain to the substrate and also increasing complexation. There was no shift in optimum pH 7.5 on adding HP-β-CD. Optimum temperature for papain was 30^{o}C and temperature stability was achieved till 55 °C, in presence of HP-β-CD. Complexes of different ratios were prepared from 1:10 to 1:200 by physical mixing, solvent evaporation and neutralization, and assayed. By physical mixtures, the enzymatic activity was found to be enhanced compared to papain alone and optimum was observed at 1:40. The free activity was reduced gradually in alkaline pH, but inclusion complex preserved its activity even in alkaline pH, and extreme temperatures. Inclusion complexes were analyzed by IR and bands showed the presence of enzyme and HP-β-CD in the complexes.

KEYWORDS: Enzyme, Papain, Hydroxy Propyl β- Cyclodextrin, Enzyme activity, Inclusion complexes.

INTRODUCTION

Several factors affect the progression of an enzymatic reaction. A few are: enzyme concentration, substrate concentration, temperature, pH, and the presence of inhibitors or activators [Robert A, Mark.F]. Extreme pH values (high or low) and temperatures generally result in complete loss of activity for most enzymes [Vyas SP, Current Index]. The clinical applications of enzymes are still limited by a number of factors, such as availability of enzymes at site of interest, which is generally very poor unless the high therapeutic dose is administered, this is often undesirable. The others are: sensitivity of enzymes to the action of various natural inhibitors, immunogenicity, destruction of enzymes by the action of endogenous proteases, *In vivo* inactivation and their fast clearance, and high cost and poor availability of pure enzymes [Jain N.K]. In order to resolve these problems, the inclusion of enzymes can be attempted with success. Such a technique makes it possible to conserve dose of enzymes, avoid degradation of enzyme *in vitro* and *in vivo*, prolong enzymatic activity at the site of interest, and reduce undesirable side effects. Inclusion complex is formed when a compound possessing an intramolecular cavity of molecular dimension, interacts with a small molecule that can enter the cavity [jain NK, Rajeswari challa]. The macrocyclic molecule is called the "host" and the small included molecule is called the "guest". The binding of "guest" molecules within the "host" cyclodextrins is not fixed or permanent, but rather in dynamic equilibrium, thereby affording an ease of assembly and disassembly. Binding strength depends on how the "host-guest" complex fits together and on specific local interactions between surface atoms [N. Pariot]. Complexes can be formed either in the solution or in the solid state. While water is typically the solvent of choice, inclusion complexation can be accomplished in co-solvent systems and with few aqueous miscible solvents. The three dimensional structure of the cyclodextrins (CD) provides a hydrophobic cavity and hydrophilic exterior microenvironment that a suitable size molecule enters and get included. No covalent bonds are formed and in aqueous solution the complexes are readily dissociated. Free drug molecules are in equilibrium with the molecules bound within the CD cavity. The sequestration of hydrophobic drugs inside the cavity of the CD can improve their solubility, stability,

the rate and extent of dissolution of drug [Raymond C, Kanaka Durga]. Various complexes with different ratio of drug to CD molecules can be attempted, depending on the type of CD used the size and physico-chemical characteristics of the drug molecule. CDs have primarily been applied as solubilizer for lipophilic drugs to enhance their bioavailability and/or reduce adverse effects after oral, parenteral or other routes of administration [vyas SP]. Physical stabilization can be achieved through inclusion complexes.

Present investigation report the preparation of papain – HP-β-CD complexes by different methods and to characterize them [Ramnik singh, Gustavo]. For the present work, proteolytic enzyme papain was chosen. HP-β-CD was used to formulate into inclusion complexes. It was expected to stabilize the papain and may act as immobilized enzyme to produce prolonged action [Irhan].

MATERIALS AND METHODS

The papain (1:2000 IU) was the gift sample from Suzikem Drugs Pvt.,Ltd., Hyderabad, hydroxyl propyl - β- cyclodextrin (HP-ß-CD), Casein (soluble), Tyrosine, Folin & Ciolcaltea's reagent were from SD Fine Chem, Mumbai. All glass double distilled water was used.

METHODS

Standardization of tyrosin

Since tyrosin was released from casein on the action of papain, the tyrosin standard curve was drawn for verifying the linearity between the absorbance and concentration [Worthington]. The tyrosin concentrations in the range of 0.02 to 1.12 mg were prepared and Folin reagent was added. The blue color was estimated at 660 nm using UV Visible spectrophotometer (Shimadzu, Japan).

Enzymatic activity

Papain enzymatic activity was determined by reported method using casein as a substrate by Folin phenol method [Worthington]. As a ready reference, the method was briefly described. The casein concentration was maintained constant (5 mg/mL) and the concentration of papain was varied from 1 to 5 mg. The casein solution was prepared by mixing 50 mg of casein in 25 mL (2%) of 50 mM potassium phosphate buffer. Gradually solution temperature was increased to 80-85 °C (was not boiled) with gentle stirring for about 10 minutes, until a homogenous dispersion is achieved. Two mL of casein solution was taken in each test tube and increased concentrations of papain were added and solutions were maintained at 37 °C for 60 min (incubation time), then 2 mL of trichloro acidic acid was mixed and incubated for 30 min at 37 °C to stop the reaction. From the reaction mixture, 2 mL filtrate was taken to another set of test tubes to which 5 mL of 500 mM sodium carbonate solution and one mL of Folin regent was added and incubated at 37 °C for 30 min for color development. The absorbances of the solutions were measured at 660 nm against the blank.

Saturation status of substrate levels

There was a need for verifying the saturation of enzyme with respect to substrate [Robert]. Therefore, casein concentrations were varied from 5 to 50 mg. The papain concentration was fixed at 4 mg in the solution. With these concentrations, the enzymatic activity was evaluated as discussed earlier.

Enzymatic activity of papain-Influence of hydroxypropyl ß cyclodextrin

The enzymatic activity was evaluated for solutions containing papain (4 mg) and increasing concentrations (0 to 4 mg) of HP ß-cyclodextrin.

Enzymatic activity of papain

Standard phosphate buffer solutions [IP] were selected in the pH range from 2.0 to 11. The casein (10 mg) was dissolved in buffer solutions. The papain concentration (4 mg) was added and the enzymatic activity was evaluated and compared with free enzyme and varying concentrations of HB- ß-CD (1-5 mg) at different pHs.

Effect of temperature

Enzymatic activity of papain

The temperature ranging from 25 to 60 °C were selected. The papain and casein concentrations were kept constant (4 mg and 10 mg respectively). HP- ß CD concentration was varied from 1 to 5 mg and enzymatic activity was studied and compared with free enzyme as per the procedure.

Preparation of solid inclusion complexes

Solid complexes were prepared by 3 different methods namely physical blending, solvent evaporation, and neutralization [Jain NK]. Physical mixture was prepared in the molar ratio of 1:10 to 1:200 between papain and HP-β –CD by mixing in a mortar for 2 hours with pestle. The powdered physical mixture was then stored at room temperature in a dessicator. In solvent evaporation method, papain – HP-ß-CD complexes were added in different ratios from 1:10 to 1:200 in common soluble solvent (water 10 mL). The solvent was removed at reduced pressure using a rotary evaporator at 60 °C and placed in dessicator for further drying. In neutralization method, papain–HP-ß-CD complexes were prepared by placing enzyme and cyclodextrin in different ratios (1:10 to 1:200) in 0.01 N hydrochloric acid and titrated with 0.01 N sodium hydroxide until precipitate was obtained and placed in dessicator for drying.

Characterization of solid inclusion complexes

Chemical estimation of papain in inclusion complex

Spectrophotometric studies were carried out by dissolving the solid complexes in distilled water (5.0 mg). The solutions were analyzed for papain and its activity in the inclusion complex, as reported earlier [Ramkin Singh].

Enzymatic activity of solid complex

Solid complexes (5.0 mg) were dissolved in water and enzymatic activity was estimated to confirm the activity of papain, as per the procedure given in enzymatic activity.

IR Spectrophotometric assay of complexes prepared by solvent evaporation

The complexes were subjected for IR analysis and bands were obtained.

Statistical methods of analysis

MS EXCEL 2007 was used for evaluating the statistical parameters, mean, standard deviation, regression analysis and graphs.

RESULTS AND DISCUSSION

Enzymatic activity of papain

The estimation of tyrosin was pre-requisite for correlating the reactivity of substrate in the enzymatic analysis. On account of enzymatic activity of papain, casein produces tyrosin. Therefore, tyrosin was separately analyzed, by producing color complex with Folin & Ciolcalteas reagent at 660 nm. The standard plot was linear (R^2 = 0.996) and Beer-Lambert law was obeyed in the range of 0.02 to 0.12 mg. The regression equation was y = 8.6577x + 0.0092, and was utilized for calculations. The enzymatic activity of papain was analysed at varying concentrations of papain (0.125 mg to 0.625 mg) [worthington]. The results were reported in Figure 1. The concentration - activity of enzyme showed a linear relationship, which indicated that the working substrate concentration adequately reflected enzymatic activity. The results indicated the suitability of the procedure for analysis. The activity of papain was nearer to the manufacturers report (1:2000 IU) (COA of company).

Effect of substrate concentration

Having established the enzyme assay method, the mid point of papain was fixed at 0.5 mg. The concentration of casein was varied (5 mg to 60 mg), in order to verify level of substrate. The casein concentration-enzymatic activity profile was given in Figure 2. The exhibited saturation level at 35 mg, beyond this, the casein showed inhibitory effect. The concentration of the casein was fixed at constant 10 mg, which was in linear trend.

Effect of HP-ß-CD on the enzymatic activity of papain

The concentration of papain was kept constant (0.5 mg) and the amount of hydroxylpropyl ß-cyclodextrin was varied from 0.2 to 1.0 mg. The absorbances data were analyzed and calculated in terms of enzyme activity (presence/absence of HP-ß-CD based on International Units) and reported (y axis of Figure 3). These results indicated the *prima facei* evidence of some type of interaction, i.e., formation of inclusion complexes *in situ* (in solution). Further as the concentration HP-ß-CD was increased the activity was decreased linearly in the work range of 1:1. This indicated that active sites responsible for enzymatic activity were not exposed for reaction with casein. As the concentration of HP-ß- CD was increased, more quantity of enzyme was incorporated into the complex.

Effect of pH

Effect of pH on the enzymatic activity of papain

Normally, the activity of papain was reported at pH 6.5 [Worthington]. The pH range was spread from 2 to 11 (Figure 4). In the present work, the enzymatic activity of papain was found to be optimum at pH 7.5. The pH (2-11) dependent activity of papain was studied in the presence of HP-β-CD. The optimum pH remained at 7.5, after forming complex with HP-β-CD. As the pH of the medium was increased, enzyme started losing its activity when present alone, whereas complexed enzyme retained its enzyme activity. At this level, the ratio of papain to HP-β-CD was 1:4 (1 mg to 4 mg). Beyond 4 mg of HP-β-CD, the enzymatic activity was decreased. Thus the optimum concentration of HP-β-CD was found to be 4 mg.

Effect of temperature

Effect of temperature on the enzymatic activity of papain

For evaluating the activity, the temperature was reported as 37°C for papain. In this study, the temperatures were varied from 25 to 60°C and the activity was found to be optimum at 30ºC (Figure 5), at papain concentration of 4 mg. In the presence HP-ß-CD, the enzymatic activity was reported (Figure 5). The profiles indicated that the presence of HP-ß-CD the activity of papain was enhanced till 55ºC.

Evaluation of complexes prepared by physical mixing

The DSC behavior of cyclodextrin-papain inclusion complex indicated higher thermal stability, when papain – ß cyclodextrin ratios were higher (1:100 etc). Such effects were explained by folding of the protein molecules accommodated in the sugar moiety. However their ratios were not confirmed by the evaluation of enzymatic activity [R]. The present work reported enahanced enzymatic activity at 1:40 ratio, suggesting the favourable folding that makes the sites active for enzymatic activity. The solid complexes of physical mixtures were evaluated for the enzymatic activity of papain. The complexes weight equivalent to 5 mg/mL of papain were taken and dissolved in water. This is further used for conducting assay. Enzymatic activity was reported in Figure 6. Activity was found to be optimum with 1:40 ratio physical mixing. The behavior of enzymes in the inclusion complexes exhibited considerable variation (non uniform trend) at higher temperature. In other words the folding and unfolding variations are governed by temperatures.

Evaluation of complexes prepared by solvent evaporation method

The bands in the IR spectra had shown the characteristics, confirming the presence of enzyme and cyclodextrin in all ratios of complexes (Table 1).

FIGURES

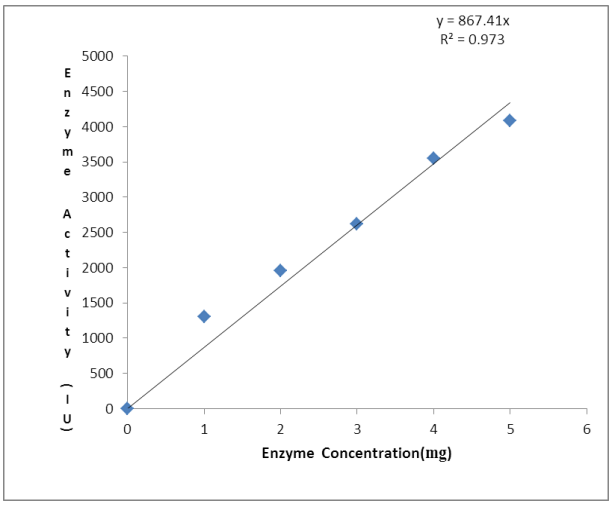

Figure 1: Enzymatic activity of papain.

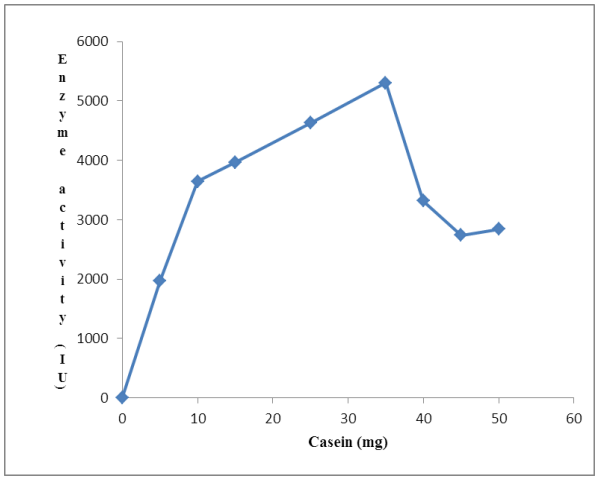

Figure 2. Effect of substrate (Casein)on enzymatic activity of papain.

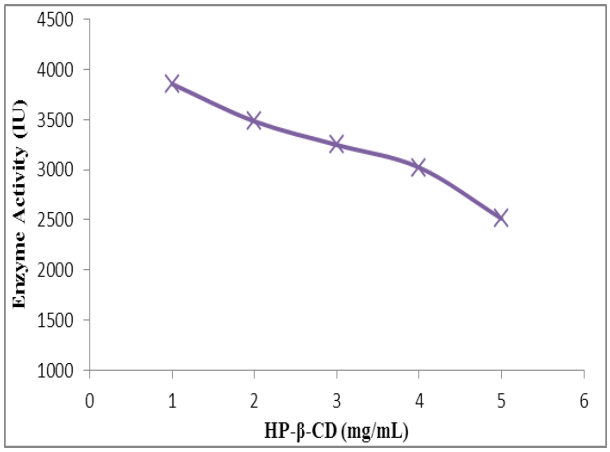

Figure 3. Enzymatic activity of papain in presence of HP-β-CD.

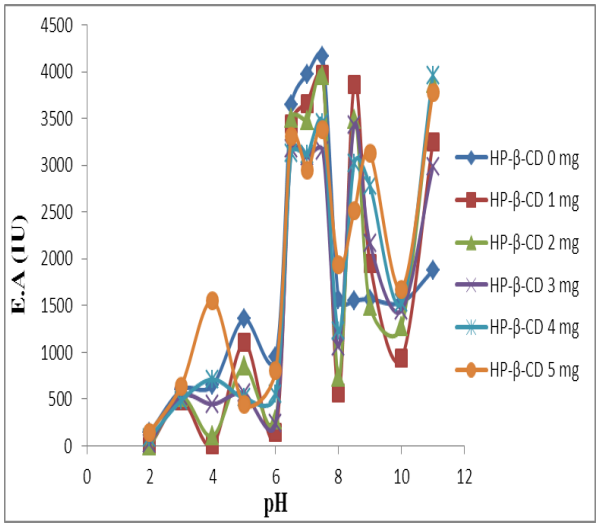

Figure 4: Enzymatic activity of papain at increasing concentrations of HP-β-CD with different pHs.

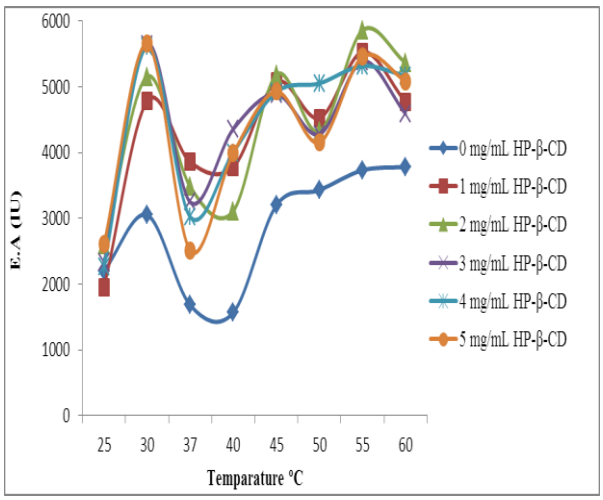

Figure. 5: Enzymatic activity–temperature profile of papain in presence of HP-β-CD.

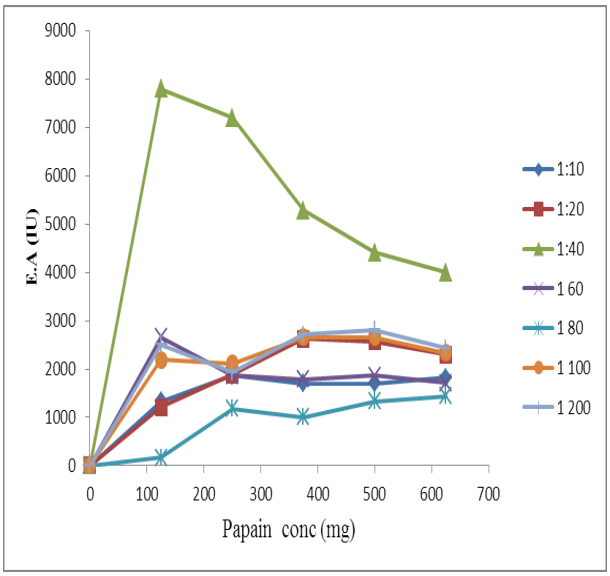

Figure. 6: Enzymatic activity of different ratios of papain-HP-β-CD complexes prepared by physical mixing.

Table 1. IR peaks in complexes observed

SL No	Functional group	Experimental range (wavenumber cm^{-1})	Theoretical range (wavenumber cm^{-1})	Comments
1	Free OH	3300-3100	3560-3500	Presence of β cyclodextrin
2	Bonded OH	3300-3100	3300-2500	Presence of β cyclodextrin
3	Phenolic C-O stretching	1580-5110	1410-1310	Presence of papain
4	Aromatic C-H stretching	3200-3100	3030	Presence of papain
5	N-H stretching		3480-3070	Presence of papain
6	C-O stretching	1700-1600	1690-1650	Presence of papain
7	Amide deformation bending	1580-1510	1570-1510	Folding of papain in the cavity of cyclodextrin
8	Amino acids NH_3 stretching		3130-3030	Presence of papain
9	Amino acids NH_3 Bending	1580-1510	1660-1610	Presence of papain
10	COO stretching	1580-1510	1600-1560	Presence of papain

CONCLUSION

The report demonstrated the formation of inclusion complexes of papain (macromolecule) with HP-ß-CD. The result was decreased enzymatic activity of papain. HP-ß-CD concentration dependent decrease in enzymatic activity was observed. This behavior was observed in the range of 1:1. Ratio of papain to HP-ß-CD in 1:40 complex showed enhanced activity, and beyond this ratio there was no increase in activity of papain. The influence of pH on activity of papain was studied from 2 – 11alone and in the presence of HP-β-CD. As the pH increased, enzyme lost its activity gradually when present alone, whereas complexed enzyme retained its activity even at pH of 11 at ratio of papain to HP-β-CD of 1:4. The enzymatic activity of papain was found optimum at 30 ºC. As the concentration of HP-β-CD increased the activity of enzyme retained up to 55ºC. Thus complexation with HP-ß-CD was beneficial in the design of formulations.

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