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(Research Article)

Complexation and Characterization of ∝-Amylase with Hydroxypropyl ß- Cyclodextrin

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ABSTRACT

Drug-β cyclodextrin inclusion complexes had been an important tool for improving the solubility and bioavailability of drugs. The aim of the present research was to exploit the feasibility by using complexed enzymes (therapeutic proteins) as an alternate method for immobilization. Such complexed product was assumed to enhance the enzymatic activity. In the present study, α-Amylase (digestive enzyme) is used along with hydroxyl propyl β-cyclodextrin (HP- β-CD) to improve stability, solubility and bioavailability of enzyme. In the present study, different molar ratios of α-Amylase and cyclodextrin were prepared with different contact period between guest and host molecule to allow complexation, and interference of HP- β-CD on enzymatic activity of α-amylase was also studied. The prima facei evidence indicates that enzyme activity was decreased, when α-amylase was complexed with increasing concentrations of HP-β-CD. Solid inclusion complex was prepared by precipitation method at isoelectric point (pH 4.6). The complex was characterized by UV spectrophotometric and FT-IR technique. The presence of α-amylase and HP-β-CD in the complex was observed, it indicates that the enzyme activity was decreased in presence of HP-β-CD. High temperature studies, acid media studies (mimicking GIT) and accelerated studies need to be explored for concluding the role of inclusion complexes in the therapy.

Keywords: α-Amylase, Enzyme, HP-β-CD, Inclusion complexes.

INTRODUCTION

Enzymes have been widely used in medicine, fermentation, and cosmetics as they are potential active ingredients due to their pharmacological and therapeutic activities. [1] These therapeutic proteins have been extensively investigated for increased stability and enzymatic activity. Most important difference between the conventional drug entities and peptide/protein drugs is their stability profile. [2] The high physical and chemical instability presents peculiar difficulties in the purification, separation, formulation, storage and delivery of these compounds in to the blood circulation. Chemical instability results in the generation of a new chemical entity, by bond formation or cleavage.[2, 3] Changes bought about by physical and chemical degradation always lead to a loss of biological activity, and most of the enzymes lose their biological activity at room temperature in a short period, limiting their application in pharmaceutical products. [1] So an efficient and reliable stabilizing method must be developed to allow applications of this enzyme in pharmaceutical and biological formulations. Complexes can be formed either in solution or in solid state. [4]

Cyclodextrins are of current interest and were attempted with a variety of drug molecules for improving the dissolution, bioavailability, masking the unpleasant taste, converting liquids into solids, reducing irritation of eye in ophthalmic preparations. [5, 6] Enzymes have several limitations, such as stability, narrow range of pH for activity, sensitivity, inactivation and fast clearance, when administered orally.[2] The advantages of cyclodextrin inclusion complexes can be extended to enzyme preparations also.[7, 8] So far small molecules were incorporated into inclusion complexes for improved dissolution stability and bio availability of the enzyme.[9] Since these oligosaccharides are able to form complexes with different molecules (including proteins), and modify their physical, chemical and biological characteristics, [10] allowing the enzyme remain active for extended period of time. Probably this methodology offers alternative to immobilized enzyme systems.

So in the present study, α-Amylase is used along with HP-β-CD to improve stability, bioavailability and enzymatic activity of enzymes.

MATERIAL AND METHODS

Materials

α- amylase and hydroxyl propyl β cyclodextrin (HP-β-CD) were procured from Suzikem Drugs Pvt., Limited, and

Hyderabad. Starch (soluble) and maltose were procured from SD Fine Chem. Mumbai.

Standardization of Maltose

Maltose was released from starch on the action of α-amylase, the maltose standard curve was drawn for verifying the linearity between the absorbance and concentration.[11] The maltose concentrations in the range of 0.4 to 2.0 mg were prepared and 3,5 dinitro salicylic acid (DNS) was added.The orange color was estimated at 540 nm using UV Visible spectrophotometer (Shimadzu 1700, Japan).

Enzymatic Activity of α-Amylase

α-Amylase enzymatic activity was determined using starch as a substrate by DNS method. [11] The starch concentration was maintained constant (0.6 mg) and the concentration of α-amylase was varied from 0.2 to 1.0 mg. The starch solution was prepared in phosphate buffer at pH 6.9 [12], α-amylase was mixed and solutions were maintained at 20 °C for 3 minutes, followed by DNS reagent. The reaction mixture was boiled in water for 15 minutes to stop the reaction. The reaction mixture was cooled to make the volume up to 9.6 mL. The absorbance of the resultant solution was measured at 540 nm against the blank.

Effect of Incubation Period of Enzyme and Substrate

According to the reported procedure in the literature[11], the α-amylase and starch (substrate) were incubated for 3 minutes at 20 ºC (pH 6.9). The incubation period was enhanced in order to verify the effectiveness of the enzyme at prolonged period (stability). The incubation period of α-amylase and starch were varied as 3 minutes, 5 minutes, 10 minutes, 15 minutes and 20 minutes at 20 ºC, the procedure remained the same as reported in the assay. After completing the incubation period, DNS reagent was added to stop the reaction. The absorbance of the resultant solution was measured at 540 nm against the blank.

UV Spectral Interference of HP-β-CD with α-Amylase

The analysis of α-amylase was done in the presence of HP-β-CD. For this purpose, α-Amylase (0.00001M), HP-β-CD (0.0001M), mixture of α-amylase (0.00001M) and HP-β-CD (0.0001M) solutions were prepared from the stock solutions.[13] These solutions were scanned for UV absorption pattern between 200-400 nm.

The concentration of α-Amylase (0.00001 M) was kept constant in all sample solutions, concentration of ß-cyclodextrin was increased (0.0001M to 0.02M) and volume of the solution was adjusted with double distilled water up to 10mL. The resultant solutions were scanned under UV spectrophotometer (200-400 nm) to know the interference of increased concentrations of HP-β-CD on α-amylase.

Effect of Contact Period on Complexation of Enzyme and HP-β-CD

α-Amylase and HP-β-CD were mixed at equal molar concentration and contact periods for the resultant solutions were kept for 1, 2, 3, and 4 h to allow the enzyme to get entrapped into the cavity of HP-β-CD. From this stock solution the samples were withdrawn for every 1h up to 4 h. The enzymatic analysis was repeated using starch as a substrate. The incubation period of α-amylase and HP-β-CD stock (complex) solution with starch was kept further at 3 minutes, 5 minutes, 10 minutes, 15 minutes and 20 minutes at 20 ºC. After incubation period, the reaction was stopped with addition of DNS reagent. The absorbance was measured UV spectrophotometer at 540 nm.

Preparation of Solid Complex of α-Amylase with HP-β-CD

Equimolar concentration of α-amylase and HP-β-CD were prepared in water, hydrochloric acid (0.1 N) was added until no more precipitate was formed[4] and pH of the resultant solution was measured. The sample was subjected to cold centrifugation [Micro centrifuge, Hyderabad] for 30 minutes at 20 °C and 1000 rpm. The supernatant liquid was decanted and the precipitate was dried at 25 °C for 3 days in a desiccator. The precipitate was triturated in a mortar to a fine powder. α-Amylase and HP-β-CD complex was formed as light yellowish-white precipitate and analyzed for its characterization.

Spectral Analysis of Solid Complex

The α-amylase solution and solid complex solutions were scanned under UV spectrophotometer[14] (200-400 nm). FTIR [Shimandzu, Toshwin, Mumbai] spectral studies were carried out for α-amylase, HP-β-CD and the solid complex to know the interference of HP-β-CD on α-amylase.

Enzymatic Activity of Solid Complex

The enzymatic activity of the HP-β-CD on α-amylase solid complex was found out as per the procedure mentioned previously [11]. The enzymatic activity of HP-β-CD on α-amylase solid complex was compared with α-amylase simultaneously.

RESULTS AND DISCUSSION

Analytical Method for α-Amylase

α-Amylase was analyzed by UV spectrophotometer (200-400 nm) which showed absorption at 276 nm. The Beer-Lambert law was obeyed in the range of 0.2-1.0mg/mL (R^2=0.9981).

Enzymatic activity was also found out for α-amylase, and results showed that enzymatic activity was increased with increasing concentration of α-amylase. As linear relationship was obtained for increasing concentrations of α-amylase it indicates that substrate had not reached to saturation level hence concentration of starch (substrate) could be fixed at 0.6 mg, which will not affect the enzymatic activity with increasing concentrations of HP-β-CD.

Enzymatic Activity of α-Amylase in Presence of HP-β-CD

The enzymatic activity of α-amylase was analyzed in presence of HP-β-CD which showed α-amylase activity was reduced linearly. The active sites responsible for enzymatic activity were not exposed for interaction with substrate. As concentration of HP-β-CD increased, α-amylase was increasingly involved in the inclusion complexation. It was assumed that mixture of α-amylase and HP-β-CDs gave *in situ* formation of inclusion complexes.

Effect of Incubation Period of Enzyme and Substrate

In order to find out, time required for enzymatic activity incubation period of enzyme and substrate was increased from 3 minutes up to 20 minutes. The results showed that

proposed 3 minutes incubation time was adequate to complete the reaction. Similar experiments were conducted using α-amylase and HP-β-CD and incubation period was increased which showed that results were not affected in the presence of HP-β-CD. It concludes that incubation time was not affected in presence of HP-β-CD (Figure 1).

Contact Period of α-Amylase and HP-β-CD

In order to achieve equilibrium for the formation of inclusion complex, the α-amylase and HP-β-CD were kept in contact with each other up to 4 h. The incubation period also varied from 3 to 20 minutes for expressing the enzymatic activity (Figure 2).

As per the previous experiments enzymatic activity of α-amylase was lost within 3 minutes but in presence of HP-β-CD enzyme was stable in liquid state till 2 h (Fig 2). After 2 h enzymatic activity of α-amylase was lost even in presence of HP-β-CD.

α-Amylase, HP-β-CD and Solid Complex Characterization by UV Spectrophotometer and FT-IR

The complex was scanned under UV spectrophotometer, it showed λ_{max} at 276 nm, and this indicated the presence of α-amylase and ratio of α-amylase and β-cyclodextrin as 1:5 (based on the absorbance) in the complex.

IR spectrum of α-amylase, HP-β-CD and solid complex showed characteristic bands at 3100-3600 cm^{-1} indicating the OH stretching vibrations (Table-1). Broadening peaks due to restriction of bending and stretching vibrations of the α-amylase was observed in IR spectra. Since α-amylase, HP-β-CD were polymers, it was difficult to assign the values clearly. A comparison of Figure 3, Figure 4 and Figure 5 indicated the prima facei evidence. Thus the complex showed the presence of α-amylase and HP-β-CD.

Enzymatic Activity of Solid Complex

The enzyme activity of solid complex was found out, which showed that enzymatic activity was decreased to five times as compared to free enzyme(Table-2). From the above result we can conclude that the ratio of HP-β-CD and α-amylase may be 5:1.

CONCLUSION

Complexation of α-amylase with β-cyclodextrin was attempted to understand the effect of inclusion complexation on the enzymatic activity. α-Amylase enzymatic activity was established and standardized. As the concentration of α-amylase increased, the enzymatic activity was increased, indicating that the substrate (starch) is more than excess (not saturated), that was required for enzymatic activity.

Enzymatic activity of the α-amylase was decreased in presence of β-cyclodextrin. In other words, the active sites responsible for enzymatic activity were not exposed from the inclusion complex. α-Amylase β-cyclodextrin inclusion complex was prepared by precipitation method at isoelectric point (4.6) which may be the suitable method for the preparation of inclusion complexes of α-amylase and β-cyclodextrin. The complex was characterized by UV and FT-IR which showed the presence of α-amylase and β-cyclodextrin in the complex. Since the enzymatic activity was reduced in presence of β-cyclodextrin, the other facet, i.e., stability of α-amylase need to be evaluated at different pH values including gastric pH of 1-3, and different temperatures. Further stability study of the dosage form needs to be investigated.

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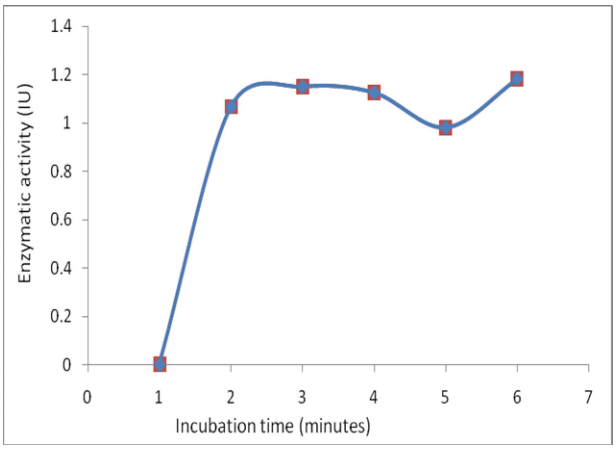


Figure 1: Effect of incubation time of α-amylase and starch on enzymatic activity

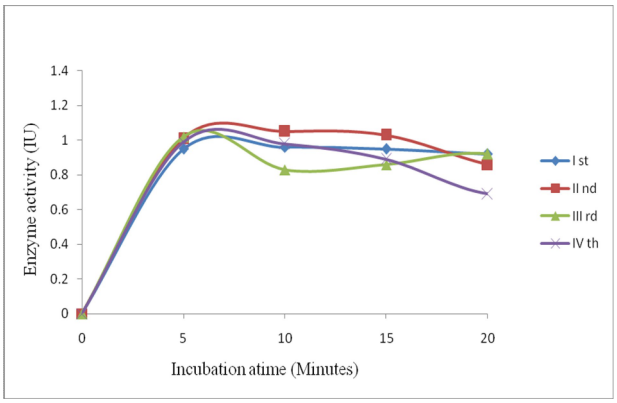


Figure 2: Effect of incubation time on the enzymatic activity at different contact times (α-amylase and β-cyclodextrin) of 1, 2, 3, 4 h.

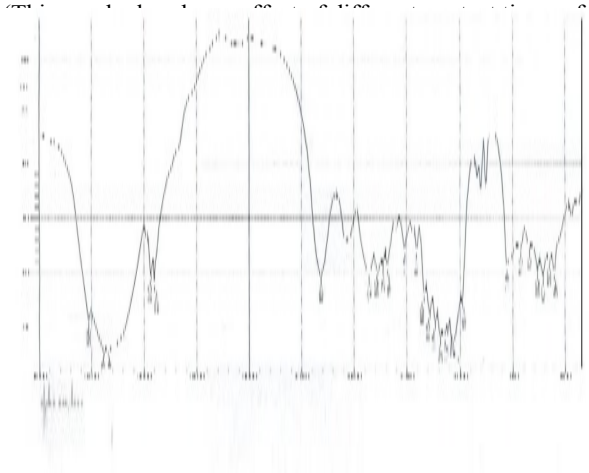


Figure 3: FT-IR spectra of α-amylase

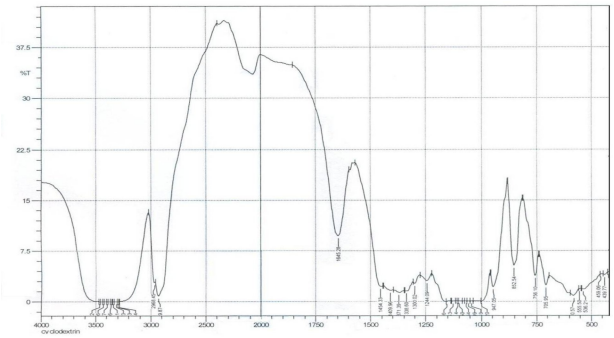
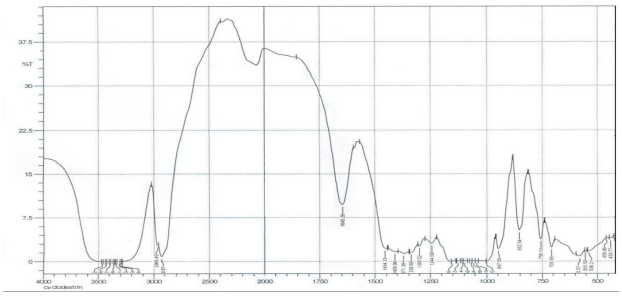


Figure 4: FT-IR spectra of β-cyclodextrin



Figur 5: FT-IR spectra of Solid complex

Table 1: Comparison of IR spectra of α-amylase and HP-β-CD with the spectra of inclusion complex and their inference.

Components	Melting point	Groups	Frequency	Inference
α- Amylase	58-60 °C	CH_2 NH CO-NH	2931, 3394 1656	Presence of α-amylase
β-Cyclodextrin	275-278 °C	CH_2 OH	2968, 3500	Presence of HP-β-CD
Inclusion complex	82-83 °C	Frequency shift		Presence of both α-amylase and HP-β-CD
		NH, C0-NH	3394 to 3354 cm-1 1656 to 1654 cm-1	

Table 2: Comparison of enzymatic activity of solid complex that of with α-amylase.

Sl No.	Concentration (mg/ml)	Enzymatic activity
α-Amylase		
1	0.2	0.124
2	0.4	0.192
3	0.6	0.223
4	0.8	0.262
Solid complex		
5	1	0.123

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