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Compressibility studies of α- Amylase

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Abstract

Background: Proteins possess greater biochemical and structural complexity compared to conventional drug based pharmaceuticals. In the present study, we investigated the effect of compression force on the tablet properties, primarily the enzyme activity and the percolation threshold to have more information of behaviour of - Amylase powder under compaction along with detection of mixture range in which enzyme is protected by the excipients.

Results: The results showed that carrageenan, tragacanth and agar provided the maximum protection to enzyme activity compared to microcrystalline cellulose and dicalcium phosphate dihydrate on compaction. However stability studies indicated the highest loss of enzyme activity with carrageenan, tragacanth and agar. The compressibility studies of different binary mixtures of - Amylase with microcrystalline cellulose indicated that - Amylase behaves like a brittle substance. The application of percolation theory on the relationship between the critical density as a function of enzyme activity and mixture composition revealed the presence of percolation threshold for binary mixture. - Amylase – microcrystalline cellulose mixture composition showed significant percolation threshold at 37.23 % (w / w) - Amylase loading.

Conclusion: Microcrystalline cellulose provided higher protection during stability study. However, higher concentrations of microcrystalline cellulose, probably as dominant particles do not protect the enzyme with their plastic deformation. Below the percolation threshold i.e. 37.23 % (w / w) - Amylase amount in mixture with plastic excipient, activity loss increases strongly because of higher shearing forces during compaction due to system dominance of plastic particles. This mixture range should therefore be avoided to get robust formulation.

Keywords: - amylase, microcrystalline cellulose, compressibility, percolation threshold.

Introduction

Among the new drug substances, the use of proteins and peptides as pharmaceuticals is steadily increasing, especially with evolution of recombinant DNA technique and advances in proteomics. Proteins possess greater biochemical and structural complexity compared to conventional drug based pharmaceuticals [1]. Thus, the formulation and delivery of proteins into stable well characterised and efficacious drug products represent significant challenges to pharmaceutical scientist [2]. Tablets are suitable dosage form for application of these materials as it provides ease of administration, metering accuracy, robustness, good stability and efficient production. However, simple compression of a bulk material, either powder or granulate, to a robust tablet is dependent on a great number of influences, mainly compression force, particle deformation and formation of adhesive forces. Therefore, the physical and chemical properties of protein can be influenced by formulation and technological factors for e.g. excipients, temperature, storage conditions, compression or shear forces [3]. Pharmaceutical tablets generally comprise a number of components, which all contribute to the final properties of tablets. However, this is a challenging task to predict the properties of the tablets based upon the knowledge of material properties of constituent substances due to the complexity and density of pharmaceutical blends. Therefore, an alternative approach would be to identify the dominant substance in terms of properties of interest, and to predict the tablet properties from properties of two or more of these dominant constituents by the application of percolation theory [4]. Many tablet properties are related to the relative density of a tablet. The percolation theory relates changes in tablet properties to the existence of critical points in pharmaceutical formulations which can be related to the percolation threshold of a compact of the formulation [5, 6]. The knowledge of these critical points and corresponding percolation thresholds is of great importance to optimise the design of pharmaceutical dosage form. Furthermore, in order to prepare robust formulations, the neighbourhood of percolation thresholds should be avoided. Otherwise a little change in the concentration of one component can cause a high variability in the properties of formulation.

α- Amylase is a major enzyme used for replacement of pancreatic enzyme during pancreatitis, cystic fibrosis or surgical removal of pancreas [7, 8]. Amylases are produced by a wide spectrum of

organisms [9]. Amylases from the source other than pancreas can also be used for replacement [10, 11]. Fungal enzymes have the advantages of high catalytic rate at moderate temperature compared to bacterial enzymes. Therefore, - Amylase from *Aspergillus oryzae* was used as model enzyme in this work.

The approach of the present work is the compaction of enzyme (- amylase) to get more information about the tablet property i.e. the enzyme activity under pressure [12, 13]. Therefore, the primary objective of the research work was detection of a percolation threshold to get more information of powder behaviour under compaction and to find out a mixture range in which the enzyme is protected by excipient.

Materials and methods

Materials

Microcrystalline cellulose (MCC) (Avicel® PH 102), dicalcium phosphate dihydrate (DCP), magnesium stearate, soluble starch, carrageenan, agar, tragacanth and 3,5 dinitrosalicylic acid (DNS) were purchased from Himedia Laboratories Pvt. Ltd., Mumbai, India. Potassium dihydrogen phosphate, sodium hydroxide (Qualigens Fine Chemicals, Mumbai, India) and Fungal - Amylase (source *Aspergillus oryzae*), sodium potassium tartarate, sodium carbonate, copper sulphate and Folin's reagent were purchased from S. D. Fine- Chem Ltd., Mumbai, India. All chemicals were used as received. Double – distilled water was used throughout the study.

Methods

Enzyme assay

Amylase activity was determined according to the procedure of Bernfeld et al. [14] after some modifications. Appropriately diluted enzyme was added to 1 ml of buffered soluble starch (1 % w/v) substrate solution (pH 6.0). After incubation at 37 C for 5 min in a shaking water bath, the reaction was stopped by the addition of 2 ml of 3, 5- dinitrosalicylic acid (DNS) reagent (1 % w/v DNS in 30 % w/v sodium potassium tartarate and 1.6 % w/v sodium hydroxide). The tubes were kept in boiling water bath for 10 min to develop colour and cooled. The absorbance was read at 540 nm in a spectrophotometer, after making up the volume to 10 ml. Amylase activity was expressed as mg of maltose liberated in 5 min by 1 mg enzyme under assay conditions. The amount of maltose formed in test samples was calculated using a calibration curve of maltose prepared by plotting absorbance versus concentration of maltose (mg/ml) in distilled water at 540 nm using UV / VIS spectrophotometer (Lab-India® UV 3000+, India).

Influences on enzyme (- amylase) activity

Enzymes are known to be sensitive to different stresses. Thus, it was important to test if temperature or excipients could influence the activity of enzyme powder.

Temperature

The influence of different temperatures on the dry enzyme powders of - Amylase was investigated. The dry powder was exposed to 30, 40, 50, 60, 70 and 80 C for 5 min i.e. the duration of compaction process. After this exposition, the enzyme activity was determined.

Excipients

The influence of various excipients, which have contact with the enzyme powder during powder compression, was investigated. Thus, dispersions of 50 % enzyme with 50 % MCC, DCP, carrageenan, agar or tragacanth was prepared in phosphate buffer (pH 6.0) and the enzyme activity was detected and compared with reference.

Preparation of tablets of enzyme with different excipients

Binary mixture of enzyme (- amylase) with MCC, DCP, carrageenan, agar and tragacanth were prepared in 1:1 ratio, respectively. The powders were mixed manually for 5 min. Samples comprising 200 mg of binary mixture powder were manually filled into a die of 8 mm in diameter (Cadmach Machinery Private Ltd., Ahmedabad) at 80 MPa compression pressure and the powders were compressed. Different batches of tablets prepared with various excipients are coded as given in Table 1. For each system 20 tablets were compressed. From the compression process only out of die data were generated.

Table 1. Different batches of tablets prepared with MCC, DCP, carrageenan, agar and tragacanth.

Excipients	Excipient : Enzyme ratio	Formulation code for - Amylase tablets
Nil	-	AP
MCC	1:1	AM
DCP	1:1	AD
Carrageenan	1:1	AC
Agar	1:1	AA
Tragacanth	1:1	AT

*AP represents tablets of - Amylase powder.

Stability study of tablets

Different batches of tablets were packed in amber coloured glass bottles and subjected to stability testing according to the International Conference on Harmonization guidelines for zone III and IV. The packed containers were kept for accelerated (40 ± 2 C / 75 ± 5 % relative humidity) and long term (30 ± 2 C / 65 ± 5 % relative humidity) stability for 6 months and 12 months, respectively. Samples kept under accelerated storage conditions were withdrawn at 0, 1.5, 3 and 6 months and - Amylase activity were estimated. Similarly, samples stored at 30 ± 2 C / 65 ± 5 % were withdrawn at 0, 3, 6, 9 and 12 months, and analysed for - Amylase activity. Visual inspection of samples for discoloration of tablet content was also done after completion of stability study.

Compaction behaviour of enzymes with MCC

Preparation of compact with MCC

Compacts of - amylase, MCC, DCP and binary mixtures of MCC and - Amylase were prepared respectively. Binary mixtures of MCC and enzyme were prepared with different fractions of constituent components. These binary mixtures consist of following amounts of - Amylase i.e. 1, 20, 40, 60, 80 % (w/w). The powders were mixed manually for 5 min. Samples comprising 200 mg of binary mixture powder were manually filled into a die of 8 mm in diameter (Cadmach Machinery Private Ltd., Ahmedabad) and the powders were compressed and decompressed without holding between the compression and decompression stages. Tablets with different densities were produced by varying the total compression pressure ranging from 40 MPa to 280 MPa. For each system and compaction pressure, 20 tablets were compressed. From the compression process only out of die data were generated.

Determination of relative density and porosity of compact

The tablet weight was measured using an electronic balance (Shimadzu AUY 220, Japan). The diameter and the thickness were measured with vernier calliper after a storage time of 24 h at 45 % relative humidity in a desiccator. From these measurements, the apparent volume and apparent density of the tablets were determined. The apparent density, ρ_a was then determined by

$$\rho_a = \text{Tablet weight} / \text{Apparentvolume}(V_{tot}) \quad \text{(i)}$$

The true density (ρ_t) of the powder was measured, using a helium gas displacement pycnometer (Type AccuPyc 1330, Micromeritics®, Bedfordshire, UK).

The relative density (ρ_r) was calculated by dividing the tablet apparent density by the true density of the binary mixture of powders used.

$$\rho_r = \rho_a / \rho_t = V_t / V_{tot} \quad \text{(ii)}$$

V_t characterises the true volume of the solid particles and therefore equation (ii) shows that the relative density is essentially a solid fraction.

The relative porosity ( ) of the compact is then calculated as

$$= (V_{tot} - V_t) / V_{tot} = 1 - V_t / V_{tot} = 1 - \rho_r \quad \text{(iii)}$$

Heckel analysis

The compaction characteristics of the powder were studied by plotting –ln(porosity) vs. compaction pressure according to Heckel equation [15]

$$\ln (1/1-\rho_r) = KP + A \quad \text{(iv)}$$

Where, ρ_r is the relative density of the compact, P is the applied pressure; K (the slope of the linear portion) is the reciprocal of the yield pressure, Py, of the material. The yield pressure is inversely related to the ability of the material to deform plastically under pressure and A is a function of the original compact volume.

Determination of percolation threshold

According to the percolation theory, critical normalised density (a function of tablet property) of various binary systems was determined by plotting enzyme activity as a function of normalised density (Apparent density / Poured density) of compact under different compression pressure [5]. The critical normalised density of various systems was determined by dividing the data in the curve into two straight lines using equation

$$y=A(m_1x+b_1)+B(m_2x+b_2) \quad \text{(v)}$$

where x is the relative density of compact, y is compaction pressure, m is the slope, b is intercept, A and B are constants. The data points around the critical value shown as a bend in the curve were attributed alternatively to the first section or to the second section. The final attribution was made considering the correlation coefficient R^2 for the overall fit.

The critical normalised density (a function of tablet property) changes in the vicinity of percolation threshold of material in binary mixture [16]. Percolation threshold of different binary mixtures of - Amylase and microcrystalline cellulose was determined by plotting critical normalised density of various systems against the percentage concentration of - Amylase in binary mixture using equation (v).

Results and discussion

Influences on enzyme activity

The influence of temperature and different excipients on the enzyme (- amylase) activity was tested. These investigations led to necessary information concerning the stability of the enzyme powder. This knowledge is important for characterisation of the enzyme powder and thus for further treatment of the enzyme powder preparation in the different studies.

Temperature

The exposure of dry enzyme powder to different temperatures for the time of a compaction process had not showed any significant change in the activity from 40 to 80 C (Figure 1). The problems are not expected to arise from temperature development in die for tablet compaction of dry enzyme powder. Temperature arise over 80 C during compression is not probable [17, 18]. Hence, possible activity loss during compression may not derive from a warming in die.

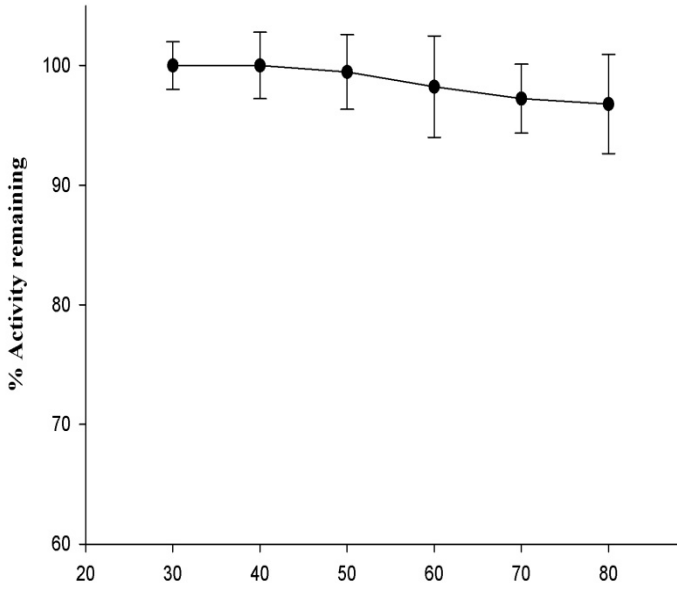

Figure 1. Effect of temperature on the stability of bulk enzyme (alpha- amylase) powder.

Excipients

The behaviour of the activity of - Amylase in the presence of various excipients was investigated and compared to the activity of α- Amylase powder as reference. The difference between the reference and the solutions of - Amylase powder and excipient, i.e. MCC, DCP, carrageenan, agar or tragacanth was not significant as the activity of enzyme remained almost same. Hence, influence of these excipients on the activity of - Amylase is not evident.

Enzyme activity of tablets

Compacts of α- Amylase along with various excipients i.e. MCC, DCP, carrageenan, agar and tragacanth showed decrease in enzyme activity respectively, when compared with the reference standards (Table 2). The greater loss in activity was observed with DCP and MCC compared to carrageenan, agar and tragacanth.

Table 2. Effect of compaction of enzyme (- amylase) with different excipients on activity of enzyme.

Formulation	% Activity remaining
AP	86.47 ± 4.13
AM	85.43 ± 3.18
AD	83.27 ± 2.56
AC	92.01 ± 3.45
AA	90.53 ± 2.67
AT	91.11 ± 4.32

*Values are mean ± SE (n = 3).

Stability studies of tablets

Tables 3 and 4 present the results of accelerated and long-term stability studies of compacts and bulk - Amylase formulations. The tablet formulations AM and AD showed around 89 % α- Amylase content on storage under accelerated conditions (i.e. 40 C / 75 % RH) for 6 months while AC, AA, AT and α- Amylase tablets showed 84 and 78 % drug content respectively (Table 3). AM and AD, however, showed around 88 % drug content when stored at 30 C / 65 % RH for 12 months against 78.58 % drug content for AP formulation. AC, AA and AT formulations showed around 85 % drug content when stored under similar conditions for 12 months. The results suggest improved stability of the enzyme on compaction with excipients. On the basis of first order degradation rate constants, the calculated t_{90} of AM, AD, AC, AA and AT at 30 C/ 65 % RH would be 341, 308, 268, 236, and 250 days respectively (Table 4). The K_{calc} / t_{90} values suggest that formulations will not provide 1 year shelf life (t_{90}) of the product and would need larger amount of overages resulting in higher initial drug concentration. The colour of α- Amylase compacts along with carrageenan, agar and tragacanth changed from pale buff to dark brown whereas α- Amylase compact formulations with MCC and DCP changed from pale buff to light brown. Thus, the stability of α- Amylase compacts along with excipients like carrageenan, agar and tragacanth was significantly lesser than compacts with MCC and DCP. The loss of enzyme activity with carrageenan, agar and tragacanth was more because of their greater moisture adsorption capacity. Thus, stability of compacts can be enhanced if protection is given against moisture to the compacts. However, greater amount of decrease in activity was observed during compaction of enzyme (α- amylase) with MCC compared to carrageenan, agar and tragacanth. Therefore, it becomes imperative to study the compression behaviour of enzyme i.e. α- Amylase along with MCC.

Compression behaviour

According to Heckel analysis, on plotting –ln (porosity) against compaction pressure, it was observed that α- Amylase and DCP behave very similarly contrary to MCC (Figure 2). Slopes of curves

(*K* values) of - Amylase and DCP were 0.0068 MPa^{-1} (R^2 = 0.995) and 0.0079 MPa^{-1} (R^2 = 0.994), respectively, whereas the *K* value of MCC was 0.0226 MPa^{-1} (R^2 = 0.980). *K* value for MCC confirms the fact that plastic substances have higher *K* values than brittle substances [15, 19]. As DCP is known to have brittle properties, α-Amylase powder can be classified as brittle substance. However the comparison of MCC with binary mixture of - Amylase – MCC in 1:1 ratio showed that there is no linear relationship between the Heckel plot of excipient alone and along with enzyme. A slight dominance of α- Amylase in the Heckel plot was seen (Figure 2). The *K* values confirmed the statement i.e. *K* = 0.0099 MPa^{-1}, 0.0226 MPa^{-1} and 0.0068 MPa^{-1} for binary mixture of α- Amylase - MCC (1:1), MCC and α- amylase, respectively.

Table 3. Stability of enzyme (α- amylase) powder tablet alone and in combination with different excipients under Accelerated Storage Conditions (40 ± 2 C / 75 ± 5 % RH)

Formulation	% Activity remaining				K ($Days^{-1}$)
	0 M	1.5 M	3 M	6 M	
AP	100.00 ± 3.20	95.87 ± 2.75	86.56 ± 3.18	77.84 ± 2.87	1.39 X 10^{-3}
AM	100.00 ± 2.08	97.88 ± 2.14	94.86 ± 2.66	89.20 ± 2.32	6.35 X 10^{-4}
AD	100.00 ± 2.48	96.47 ± 2.61	93.50 ± 4.64	88.71 ± 3.16	6.66 X 10^{-4}
AC	100.00 ± 2.19	97.08 ± 3.52	91.70 ± 2.88	84.26 ± 2.32	9.52 X 10^{-4}
AA	100.00 ± 2.08	96.74 ± 2.94	90.85 ± 2.66	85.35 ± 2.68	8.80 X 10^{-4}
AT	100.00 ± 2.28	95.47 ± 3.93	91.59 ± 2.65	84.11 ± 2.67	9.61 X 10^{-4}

*Values are mean ± SE (n = 3), M: months, K_{cal} : calculated first – order degradation rate constant

Table 4. Stability of enzyme (α- amylase) tablet alone and along with different excipients under Room Temperature Storage (30 ± 2 C / 65 ± 5 % RH).

Formulation	% Activity remaining					K	t_{90} (Days)
	0 M	3 M	6 M	9 M	12 M	($Days^{-1}$)	
AP	100.00 ± 1.57	94.41 ± 1.38	90.42 ± 1.18	85.08 ± 1.35	78.58 ± 1.26	6.61 X 10^{-4}	157.45
AM	100.01 ± 2.08	97.05 ± 2.62	94.41 ± 2.05	92.12 ± 2.23	89.48 ± 1.62	3.05 X 10^{-4}	341.44
AD	100.00 ± 1.48	96.03 ± 2.43	93.36 ± 2.21	90.99 ± 2.43	88.43 ± 2.11	3.37 X 10^{-4}	308.67
AC	100.00 ± 2.19	96.01 ± 3.09	92.53 ± 2.55	89.48 ± 2.92	86.83 ± 2.46	3.87 X 10^{-4}	268.76
AA	100.00 ± 2.08	96.01 ± 2.52	92.92 ± 2.76	89.83 ± 2.58	85.19 ± 2.71	4.39 X 10^{-4}	236.78
AT	100.00 ± 2.28	95.74 ± 2.51	92.66 ± 2.75	89.58 ± 2.57	85.95 ± 2.71	4.15 X 10^{-4}	250.67

*Values are mean ± SE (n = 3), M: months, K_{cal} : calculated first – order degradation rate constant, t_{90}: time to reach 90% of initial drug concentration.

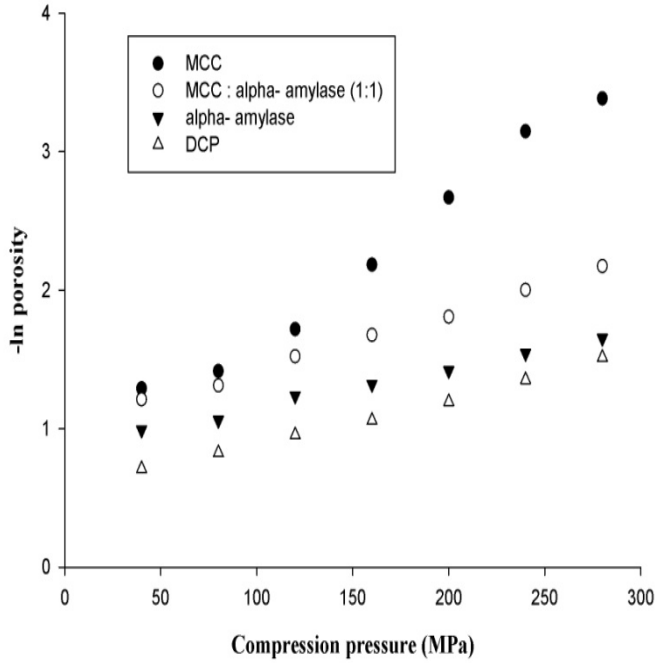

Figure 2. Heckel plots of comparison of - amylase, DCP, MCC and alpha- amylase – MCC binary mixture (1:1).

The behaviour of the enzyme under compression was further characterized by determining the activity loss at different compression pressure (Figure 3). The enzyme activity decreased steadily up to a compaction pressure of 160 MPa whereas at higher compaction pressures, the curve flattened and the degree of the activity loss decreased. Comparison with the porosity of the tablets showed that the curve also flattened slightly after the pressure of 160 MPa. The correlation coefficient *r* of activity of - Amylase with the porosity of their respective tablets showed a value of 0.984, suggesting a positive correlation. This correlation can be explained with the reduction of interparticle space, which is big in the stage of particle movement and rearrangement and diminishes in the stage of particle deformation and therefore by a destruction of the native state of the enzyme under compression. This destruction is probably linear in the stage of particle movement and rearrangement. In the stage of deformation, i.e. the region of the flattening slope, shearing forces will probably decrease as a consequence of the reduced particle movement.

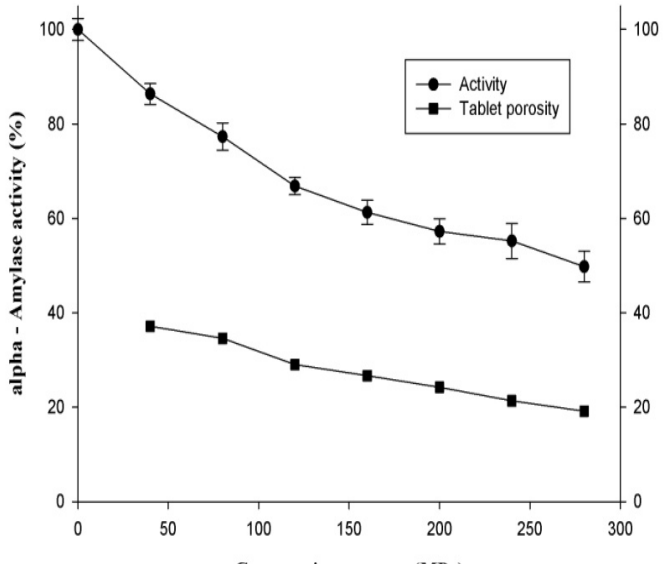

Figure 3. Relationship between alpha- amylase activity loss and porosity of tablet with increase of compression pressure.

Determination of critical density

Enzyme activity was analyzed as a function of the apparent density of compact. The apparent density of compact is a result of compaction pressure applied. Before compression, 100 % of enzyme activity was observed for the material. The apparent density of compact was normalized with the poured density of material before compression. Thus, for the normalised relative density $\rho_n = 1$, 100 % enzyme activity and for the higher values for ρ_n, the respective value of enzyme activity were measured. The relationship between activity and density of the compacts analysed with different composition of binary mixture of excipient and enzyme (α- amylase) is shown in Figure 4. The curves so obtained with different binary mixtures were approximated with two linear sections using equation (v) and each section was linearised by two regression lines. The intersection of two regression lines was determined as critical normalised density. Thus for each mixture, a critical normalised density was obtained, where the enzyme activity loss becomes more important. At higher compaction pressures, the degree of activity loss is more [20]. Figure 4 also shows that the activity loss at very high pressures depends only on the final apparent density of the compact (out-of-die).

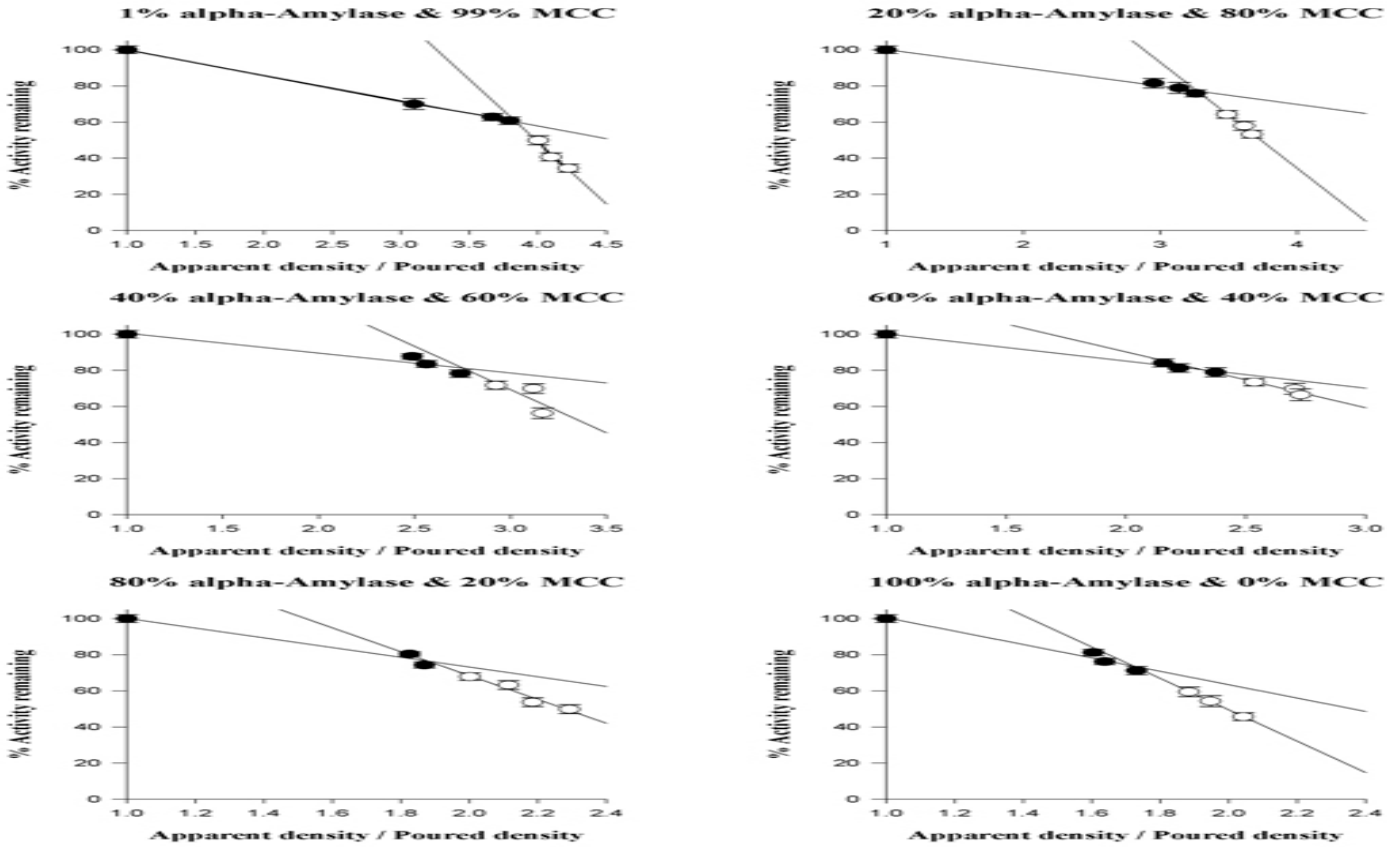

Figure 4. Relationship between alpha- amylase activity remaining (%) in tablet with density on compaction of different binary mixtures of alpha-amylase with MCC.

The detected critical normalized densities of various binary α-Amylase - MCC powder mixtures were plotted as a function of the amount of α- Amylase (Figure 5). The plot was divided into two sections with a squared correlation coefficient R^2 of 0.999 for section 1 and 0.964 (Figure 5), respectively. The point of intersection i.e. 37.23 % (w / w) (Figure 5) indicates the critical concentration of α- Amylase for binary mixtures with MCC, respectively. This critical α- Amylase concentration can be interpreted as the percolation threshold of these binary mixture system. It defines the point where the system dominance of MCC is replaced by the dominance of α- amylase. Above the percolation threshold, the brittle substance (α- Amylase powder) builds a lattice. The rigidity of the lattice and the filling of pore spaces with MCC particles prevent the fracturing of the brittle particles and therefore reduce the shearing forces in the compact. This seems to be favourable and reduces the loss of enzyme activity (Figure 6).

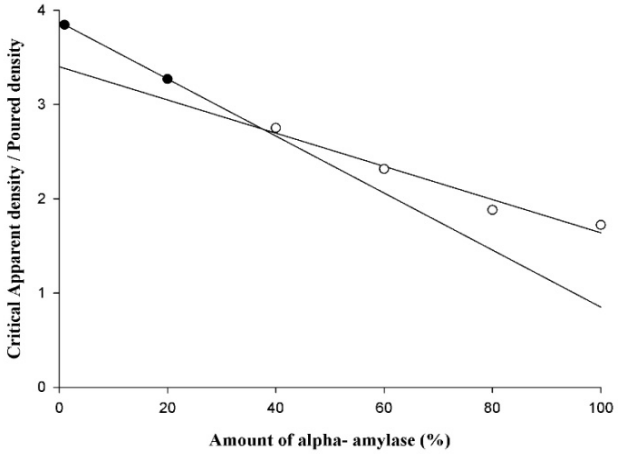

Figure 5. Relationship between critical densities of each binary mixture of alpha- amylase with MCC and the amount of alpha-amylase (%).

Below the percolation threshold, the enzyme is completely embedded in the MCC i.e. in the plastic material. As the compaction pressure applied, tablet can be compacted to higher density because of plastic flow of material. Thus tablet porosity is reduced along with increased activity loss as rigid lattice of the enzyme powder is destroyed. In addition, the shearing forces are higher with a dominance of MCC because particle movement is bigger as compared to lattice dominated by the brittle substance. This mixture range should therefore be avoided to get robust formulations because at that mixture range a sudden change in the behaviour of the system occurs. Thus, the plastic powder (MCC) does not work as the protecting substance during compression.

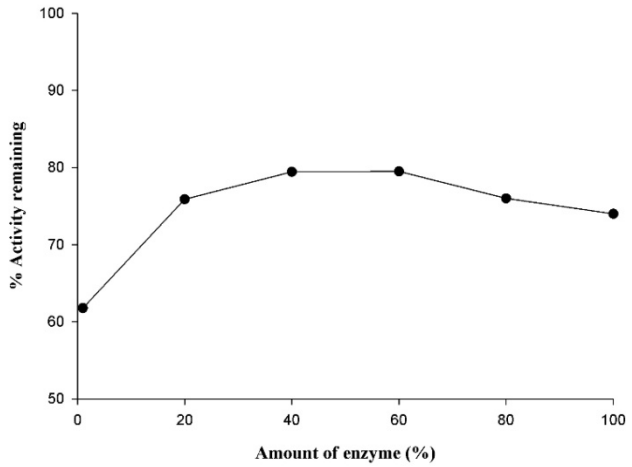

Figure 6. Relationship between percentage enzyme activity remaining and the amount of enzyme (%) at different critical density of binary mixtures of enzyme with MCC.

Conclusions

The activity of investigated model enzyme - Amylase was negatively influenced by the application of compaction pressure, whereas the extent of activity loss was dependent on the compression character of compacted substances. Compression properties of α- Amylase studied with excipients showed that enzyme powder compressed alone had greater loss in enzyme activity compared to combinations with excipients like carrageenan, tragacanth and agar. On the contrary MCC and DCP did not show protection to enzyme activity similar to carrageenan, agar and tragacanth. However, MCC provided higher protection during stability study. The compaction behaviour of enzyme with varying concentration of MCC indicated that increase in MCC content in binary mixture increased the apparent density of compact with reduced porosity. The higher concentration of MCC, probably as the dominant particles do not protect the enzyme with their plastic deformation. They do possibly crush the particles of the enzyme powder and cause high shearing forces on the enzyme. The percolation threshold, i.e. the change in the system dominance was found to be at a mixture ratio of 37.23 (w / w) of - Amylase enzyme powder. Below the percolation threshold i.e. < 40 % (w / w) for α- Amylase powder, there was steep decrease in enzyme activity. This mixture range should therefore be avoided to get robust formulations because at that mixture range a sudden change in the behaviour of the system occurs and the expected protecting effect of the plastic excipient on the brittle α- Amylase enzyme powder was therefore not found.

Authors' contributions

Manu Sharma carried out the experimental work, participated in the sequence alignment and drafted the manuscript. Vinay Sharma participated in the design of the study. Dipak Kanti Majumdar conceived of the study, and participated in its design and coordination and helped to draft the manuscript. All authors read and approved the final manuscript.

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Declaration of interest

The authors report no declarations of interest.

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