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AN OVERVIEW FOR THE EFFECTS OF LACTITOL, GELUCIRE 44/14 AND COPOVIDONE ON LYSOZYME BIOLOGICAL ACTIVITY

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ABSTRACT

Lysozyme, a model protein, was used to study the effects of excipients (lactitol, copovidone and gelucire 44/14) on the protein biological activity. Three different concentrations (0.1, 0.5 and 10 %w/v) of the excipients were used with a relatively high (10%w/v) lysozyme concentration. Analytical methods such as UV spectroscopy and fluorescence technique were used to study the effect of excipients on protein biological stability and integrity. The data obtained from analysis showed that gelucire 44/14 had a disadvantage effect on lysozyme stability as a single excipient. However, by combining gelucire 44/14 with lactitol, the protein activity was preserved. Also, the combination of lactitol and copovidone preserved the integrity and activity of 6% lysozyme to a great extent. Different excipients stabilised proteins to different degree. As conclusion, there is no excipient that works well for even one type of proteins. The most suitable excipient for each particular protein is found by extensive research studying the activity and stability of the protein in the presence of excipients. High efficacy and accuracy analytic methods should be used in order to give reliable information on protein stability and integrity.

KEYWORDS: lysozyme, lactitol, copovidone, gelucire 44/14, stability, biological activity.

INTRODUCTION

The limited stability of proteins and peptides in aqueous media is becoming the major problem in the effective improvement and commercialisation of ideal protein pharmaceutical dosage forms suitable for protein delivery. Improving proteins stability in the pharmaceutical formulations has an impact on protein-based therapeutic drugs and vaccines on world health.

Development of proteins as therapeutic dosage forms has attracted several researchers during the last decades (see for example; Qodratnama et al., 2015; Gu et al., 2015; Mudassir et al., 2015; Zhang et al., 2014; Haj-Ahmad et al., 2013; Jiang et al., 2005). Most of the critical steps in the pharmaceutical processing of proteins is to maintain proteins biological activity, folded state and stability during the pharmaceutical processing, periods of storage, transportation and administration to patients (Wu et al., 2015; Dobson, 2003; Change and Hershenson, 2002; Manning et al., 1989; Privalov, 1979). Therefore, in order to preserve the biological function of proteins, the physically and chemically properties of proteins should be controlled (Haj-Ahmad et al., 2013).

Several pharmaceutical methods were utilised for protein delivery, such systems like; niosomes (e.g. delivery of lysozyme by niosomes, see for example Haj-Ahmad et al., 2010), liposomes (e.g. delivery of insulin via liposomes, see for example Niu et al., 2011), electrospun nanofibers (e.g. bovine serum albumin, see for example Esfahani et al., 2015) and electrospryed nanoparticles (e.g. delivery of insulin, see for example Bakhshi et al., 2011) mucoadhesive tablets (e.g. delivery of oxytocin, see for example Metia and Bandyopadhyay, 2008). However, all of these pharmaceutical formulations produces dry solid product that might be affected by the moisture upon storage.

Ideally, a pure protein formulation is the supreme formulation that everyone is looking for. However, it is impossible to launch a pharmaceutical formulation that just contain the native protein due to the difficulty of protein purification, protein stability and high probability of contamination (Change and Hershenson, 2002) especially in liquid form (Malik et al., 2013). Accordingly, several attempts have documented using various excipients in order to maintain the integrity of proteins in solid form (see for example; Liu et al., 2014, Santana et al., 2014) and liquid forms (see for example; Maddux et al., 2014; Malik et al., 2013). Proteins are not stable enough to be handled as a liquid formulation, meaning high risk of protein denaturation and degradation (Bock et al., 2012).

To elucidate the impact of some additives on protein activity and conformational stability in liquid interface, it is necessary to examine the biological activity, tertiary and secondary structure stability in solution forms. A clear understanding of the protein-excipient interactions would help in the development of protein-based drugs (Kamerzell et al., 2011). Moreover, choosing the right excipient is critical to stabilise the protein during storage time.

Some excipients were reported to have a negative influence on proteins activity such as mannitol in liquid form (Rochelle et al., 2005; Kang et al., 2002).

In this work, lysozyme (a globular protein with antibacterial activity) was used as a model protein as it is well characterised in the literatures (Haj-Ahmad et al., 2013, Elkordy et al., 2008, Rosenberger, 1996 and Roxby and Tanford, 1971). The aim of this research is to study the effects of some excipients (lactitol, copovidone and gelucire 44/14) on the stability of lysozyme as a model protein in solution forms and to study the protein-excipient relationship using different excipients. Lactitol (sugar alcohol), copovidone (1-vinyl-2-pyrrolidone and vinyl acetate copolymer) and gelucire 44/14 (a hydrophilic excipient with Hydrophilic-Lipophilic Balance of 14 and a melting temperature of 44 °C (Roussin et al., 1997) were used to access the influences of these excipients on lysozyme stability at different concentrations of excipients in aqueous media. This was investigated by studying (i) the influences of each excipient on the protein stability, integrity and activity in solution forms, (ii) the influence of adding two excipients on the stability, integrity and activity of lysozyme in solution forms and (iii) the relationship between the increase of excipient concentrations and the activity of lysozyme in liquid formulation with various lysozyme concentration.

MATERIALS AND METHODS

Materials

Lysozyme was purchased from BBI Enzyme Ltd, UK. Micrococcus Lysodeikticus (lyophilised cells) and copovidone poly (1-vinylpyrrolidone-co-vinyl acetate) were obtained from Sigma-Aldrich, UK. Gelucire 44/14 was obtained from Gattefossé, France. Crystalline milled lactitol was from Cultor Food Science, USA. Sodium hydroxide was obtained from VWR International, UK. Sodium chloride was purchased from BDH Chemical Ltd, UK. Potassium phosphate, monobasic was purchased from Fisher Scientific, US. All other reagents were either of pharmacological or analytical grade.

Protein formulations with a single excipient

Solutions with different concentrations of lysozyme and excipients (lactitol, copovidone and gelucire 44/14) were prepared in 0.076M phosphate buffer, pH 6.6 according to protein: excipient weight (g) ratios. The protein: excipient ratios, w/w, were 10:0.1, 10:0.5 and 10:1 in 100ml buffer, pH 6.6. Blank solutions (containing only excipients) were also prepared for each formulation.

Protein formulations with two excipients

Protein formulations containing lysozyme and two excipients were prepared according to the protein: combined excipients weight (g) ratios as follow: 1:0.5:0.5, 6: 0.5:0.5 and 10: 0.5:0.5 in 0.076M phosphate buffer, pH 6.6 (100ml). Mixtures of two excipients (1:1) in the same buffer were firstly prepared by combing the following excipients together: lactitol and copovidone, lactitol and gelucire 44/14, and copovidone and gelucire 44/14.

Characterisation of lysozyme formulations

Protein concentration determination

Protein concentration in the prepared formulations were determined by using UV spectrophotometry (CamSpec, UK). The presence of aromatic amino acids (Tyrosine, tryptophan and phenylalanine) in the protein structure can be detected at 280 nm. A calibration plot was constructed (in lysozyme concentration range of 0.05–0.40 mg/ml) in phosphate buffer, 0.067 M, pH 6.6; the squared correlation coefficient value was 0.992.

Biological activity assay for lysozyme

Lysozyme enzymatic activity can be detected by measuring the rate of hydrolysis of β-1, 4-glycosidic linkages between N-acetylglucosamine and N-acetylmuramic acid in bacterial cell walls. Using M501 Single beam Scanning UV/Visible spectrophotometer Camspec (Biochrom, UK), the biological activity of lysozyme was assayed by following a procedure described by Elkordy et al (2002), 100ml of bacterial suspension was freshly prepared by mixing 90ml of bacterial suspension (20 mg Micrococcus Lysodeikticus added to 90ml of potassium phosphate buffer 0.067 M, pH 6.6) and 10 ml of 1% sodium chloride (NaCl) solution. The biological reaction was initiated by adding 0.5 ml of each protein solution (lysozyme formulations) in concentration of 1.5 µg/ml prepared in the same buffer to 5ml of the bacterial suspension. Lysozyme activity was determined using the following equation (Shugar, 1952):

Activity (Units/mg) = Δ450 nm/min/(0.001 x mg enzyme in the reaction) (Eq. 1)

The unit activity of lysozyme was monitored by the reduction in the absorption rate at 450 nm.

Fluorescence analysis

Fluorescence spectra were recorded using Perkin Elmer Luminescence Spectrometer (model LS50B, made in UK) following an excitation at 350nm and emission of 394nm wavelengths. A one cm quartz cell was used. All spectra were recorded using the same conditions:

excitation and emission slits were adjusted at 10nm band width and scan speed set at 500nm/min. The emission scan range was recorded between 370nm and 600nm. Fluorescence measurements were recorded for each blank solution prepared for respective formulation under identical conditions. The data obtained were processed with FL WinLab software (version 4.00.03) (Perkin Elmer, UK). Spekwin32 software, (version 1.71.6.1) was used for spectrum subtraction and to identify peak positions.

RESULTS AND DISCUSSION

Visual inspection of all protein: excipient/s formulations (Table 1) revealed some turbidity in the protein solution for the gelucire 44/14 containing samples (Fig. 1) that could be related to protein aggregation in solution forms. Accordingly, no further assessment was applied to these samples. All protein samples contain gelucire 44/14 as a single excipient exposed white gel or turbidity in the solution form. It is documented that, increasing the moisture content of gelucire 44/14 forms white gels that are composed of liquid crystalline phases (hexagonal and lamellar mesophases) are formed. This gel dissolves at 35 ºC (Svensson et al., 2004). However, combination of gelucire 44/14 with lactitol at certain ratios of the protein and excipients exhibited different results (see below).

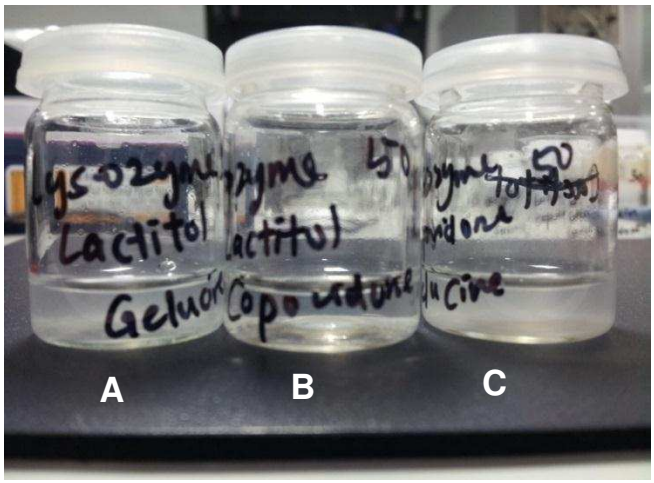

Fig. 1: Lysozyme formulations containing 10% Lysozyme and A) Lactitol and Gelucire B) Lactitol and Copovidone C) Copovidone and Gelucire. Solution A and C are omitted in the following protein characterisation procedure.

Table 1 shows the percentage concentration of lysozyme content relative to the initial concentration of lysozyme added in each formulation (10% lysozyme). For 10% lysozyme: excipient/s formulations, two samples only showed 100% of protein content preservation (0.1% lactitol and 0.1% copovidone), meaning that at this high protein concentration lactitol

and copovidone are promising excipients compared to gelucire 44/14. In the other hand, all different excipients at their different ratios (% w/v) preserved more than 90% of protein concentration either using the excipient alone or by combining two excipients in the same formulation (see Table 1). This study was conducted using ultraviolet spectrophotometry that totally depends on the aromatic amino acid residues (tyrosine, tryptophan and/or phenylalanine) to absorb the ultraviolet light at a wavelength of 280 nm (Haj-Ahmad et al., 2013). The presence of protein in a liquid environment can lead to some structural changes in these residues and might even affect the whole structure of the protein (Li and Li, 2011). Consequently, the presence of some excipients at certain concentration helped to preserve the protein content of lysozyme in the liquid form.

Table 1: **Formulations, protein content results of lysozyme preparations with and without excipients.**

composition	Protein content (%)*
10% Lysozyme with no excipients	93.5 (0.120)
10% Lysozyme with Lactitol	
Lysozyme with 0.1% Lactitol	103.7 (0.12)
Lysozyme with 0.5% Lactitol	93.8 (0.24)
Lysozyme with 1.0% Lactitol	99.0 (0.12)
10% Lysozyme with Copovidone	
Lysozyme with 0.1% Copovidone	101.1 (0.120)
Lysozyme with 0.5% Copovidone	97.0 (0.367)
Lysozyme with 1.0% Copovidone	99.2 (0.367)
10% Lysozyme with Gelucire 44/14	
Lysozyme with 0.1% Gelucire 44/14	NA
Lysozyme with 0.5% Gelucire 44/14	NA
Lysozyme with 1.0% Gelucire 44/14	NA
Combined excipients	
1% Lysozyme with Lactitol and Copovidone	107.9 (0.615)
1% Lysozyme with Lactitol and Gelucire 44/14	100.9 (0.127)
1% Lysozyme with Copovidone and Gelucire 44/14	97.2 (0.248)
6% Lysozyme with Lactitol and Copovidone	116.5 (0.248)
6% Lysozyme with Lactitol and Gelucire 44/14	96.1 (0.106)
6% Lysozyme with Copovidone and Gelucire 44/14	100.5 (0.410)
10% Lysozyme with Lactitol and Copovidone	95.1 (0.488)
10% Lysozyme with Lactitol and Gelucire 44/14	96.0 (0.495)
10% Lysozyme with Copovidone and Gelucire 44/14	99.4 (0.127)

* Values are the mean (standard deviation).

Biological activity of lysozyme is the most important parameter to define how successful are the excipients in preserving the integrity and stability of lysozyme. The biological activity results of lysozyme were expressed as a percentage ± SD relative to that of the % of

lysozyme per each formulation (refer to Fig. 2 & 3). For 1% lysozyme: excipients' formulations, the biological activity of lysozyme was preserved using a combination of lactitol and gelucire 44/14 (100% protein activity) and a combination of copovidone and gelucire 44/14 (80% protein activity) (Fig. 3). Accordingly, gelucire 44/14 as an excipient was effective in preserving the protein integrity and stability when combined with another excipients but not alone. For 6% lysozyme: excipient/s formulations: the biological activity of lysozyme was maintained by combining lactitol and copovidone in one formulation. For 10% lysozyme: excipient/s formulations, all concentrations of lactitol or copovidone used as a single excipient in formulations showed about 100% biological activity of lysozyme (Fig. 2). However, the combined excipients in the used concentration (0.5:0.5 w/w in 100ml buffer) were not successful to maintain protein biological activity at lysozyme concentration of 10%w/v (Fig. 3). The results of the biological activity assay were confirmed by the fluorescence analysis of lysozyme formulations results.

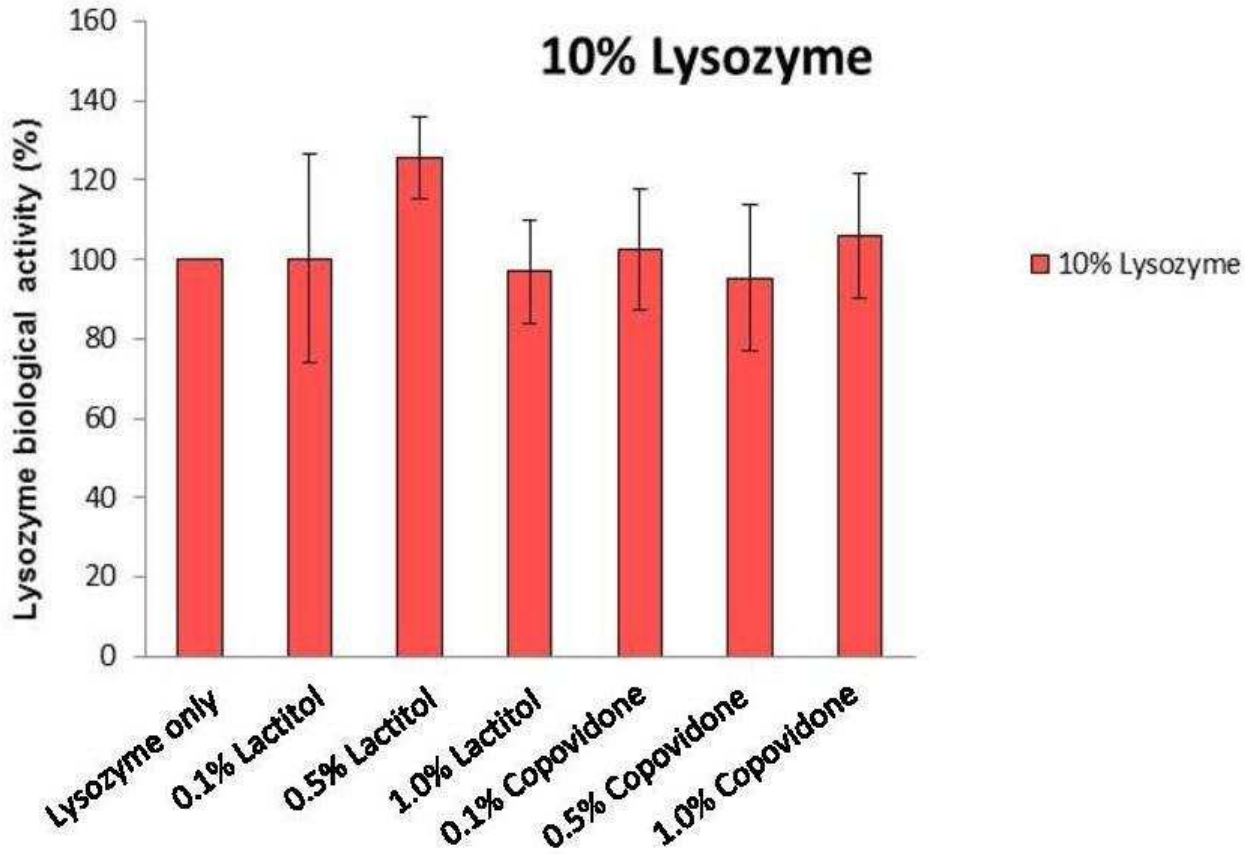

Fig. 2: Percentage lysozyme biological activity of formulations containing different type of excipient, at different concentration.

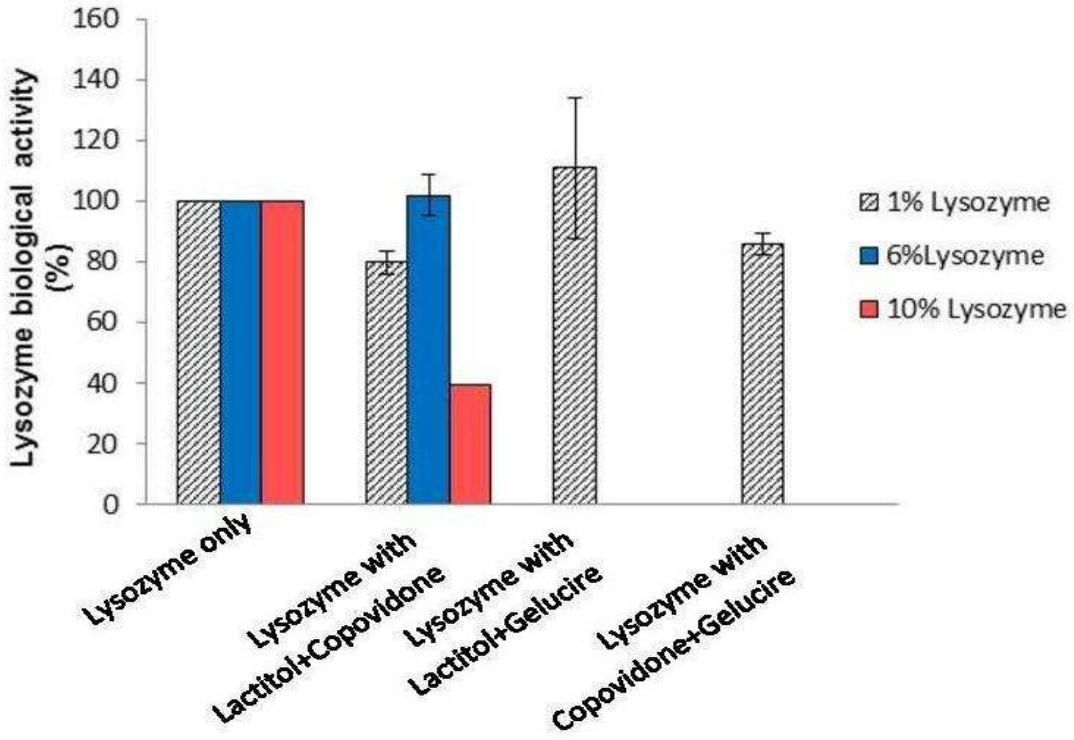

Fig 3. Percentage lysozyme biological activity of formulations contains two excipients.

Fluorescence spectroscopy analysis of lysozyme formulations was applied to monitor the influences of the used excipients on lysozyme in solution forms by identifying the changes in fluorescence intensity. For 10% lysozyme formulations, minor reduction of fluorescents intensities were observed for samples with single/combined excipients. For 10% lysozyme formulations, the data shows major increases on the fluorescents intensities for 10% lysozyme with combined excipients. The folded three dimensional structure of proteins is related to its activity. Tryptophan (Trp) is the most important amino acid residue in proteins biological activity. Lysozyme contains six tryptophan residues, three of them are located in the active site (Trp residues 62, 63 and 108) in which Trp 62 and Trp 108 are the dominant emitters (Imoto et al., 1972). Changes in the fluorescent intensity might result from a partial loosing of the tight packing around one of the Trp residues in lysozyme active site (Laurents and Baldwin, 1997), and this can justify the significant reduction of lysozyme activity in the formulations with low biological activity.

The addition of the used excipients in certain percentage to lysozyme (in certain ratio) in aqueous phase, reduced and prevented lysozyme aggregation by forming a soluble lysozyme-excipient/s complex. The possible way of preserving the lysozyme integrity by the used excipients could be by interacting with the accessible hydrophobic portion of lysozyme

through hydrophobic moiety, hydrogen bonding or van der Waals interactions, thus, increased the lysozyme solubility and reduced protein aggregation. Another suggested mechanism in which additives can preserve a fully active protein is by preserving the actual size of the binding cleft of the protein by the potency of these excipients to replace water molecules in the protein active site and make water bridges between different regions within the protein molecule. This consequently, preserves the actual size of the binding cleft of the protein (Nagendra et al., 1998).

CONCLUSION

In conclusion, lactitol and copovidone as single and combined excipients successfully maintained the biological activity and structural integrity of lysozyme in aqueous formulations. Gelucire 44/14 was only effective upon combining with other excipients such as lactitol. Different concentrations of the protein has also an impact on the formulations. Accordingly, an optimum concentration of both protein and excipient/s can be utilised to formulate a stable and active protein formulation in liquid forms.

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