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Cyclodextrin complexes of a globular protein and a lipophilic oligopeptide: the effect of structure and physicochemical properties

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Cyclosporine A (CyA) is a lipophilic oligopeptide that has a very limited solubility in water of only 0.008 mg/ml at ambient temperature. It has the ability to form inclusion complexes with cyclodextrin (CD) whose complexes can self-assemble to form aggregates. We have previously developed eye drops with CyA/CD aggregates. Our aim was to study cyclodextrin complexes of lysozyme, a small polar globular protein, and to compare the results with those obtained for CyA. We also wanted to test the stabilizing effect of CDs on lysozyme. Phase-solubility studies of various CDs were performed with CyA and lysozyme. Complexation and particle size measurements were made with dynamic light scattering (DLS) and UV. Solid drug fractions were determined. Thermal and chemical stability studies were performed on lysozyme in the presence of various CDs. Recovery of lysozyme activity in the presence of various CDs after a heat shock was determined. Both CyA and lysozyme are able to form non-inclusion complexes with CD and those complexes can self-assemble and form micro sized aggregates. In case of lysozyme the forces involved are relativity weak and the lysozyme/CD complexes dissociate upon centrifuging, however for CyA the aggregates are stronger and do not dissociate upon centrifuging. CyA is therefore suitable for eye drop preparations containing CDs for sustained drug release whereas lysozyme is not. This is mainly due to the fact that CyA forms inclusion complexes with CDs, whereas lysozyme is not able to do so due to its polar surface. The lysozyme/CD non-inclusion complexes can offer some protection against lysozymes chemical and thermal denaturation. CD can, however, form complexes with unfolded lysozyme and hamper refolding of the protein after heat shock.

1. Introduction

Over the last few decades there has been significant increase in the availability of new biopharmaceuticals (i.e. biological medical products). Peptide and protein drugs are gaining popularity in treatment of critical and threatening diseases in almost every field of medicine, mostly due to the fact that these drugs have high specificity and activity at relatively low concentrations (Frokjaer and Otzen 2005; Muheem et al. 2016; Wang 1999). In general, they are well tolerated, less likely to interfere with normal biological process or to cause side effects (Leader et al. 2008).

Peptides are relatively short chains of amino acids that are linked together via peptide bonds and have molecular weights of up to 10 kDa (Mahmood and Green 2005). An oligopeptide contains from 2 to about 20 amino acids while a polypeptide consist of a longer single linear chain of amino acids. A protein consists of one or more polypeptide chains containing more than about 50 amino acids. For proteins to be able to carry out their function the polypeptide chains need to be folded into a well-defined native structure (Vendruscolo 2012). Folding of a protein is a process where the linear polypeptide chain structure drives the protein to fold into a three-dimensional structure. This is a complex and heterogeneous process that relies on cooperation of weak, non-covalent interactions (Hartl 2011; Hendrick 1995). These include electrostatic interactions, hydrophobic bonding, hydrogen bonds and van der Waals forces (Dill MacCallum 2012). Of those interactions hydrophobic bonding is the dominant driving force for folding of soluble proteins, where about 80% of non-polar amino acids get buried within the protein interior (Wang 1999). Therefore, non-membrane proteins have a hydrophilic exterior and are water soluble. Peptides, on the other hand, do not fold and, thus, can be hydrophobic and poorly soluble in aqueous solutions.

1.1. Protein and peptide dug formulation obstacles

Therapeutic success of a protein and peptide drug dependents ultimately on the delivery of the intact drug molecules to their site of action. Unfortunately, proteins and peptides are frequently highly unstable displaying both physical and chemical instability in aqueous environments. Proteins and peptides display enzymatic instability, do not readily permeate biological membranes, are relatively rapidly cleared from the blood circulation and can be immunogenic (Irie Uekama 1999; Patel 2014). The instability of proteins in the gastro-intestinal tract, and their low permeability across biological membranes, prevents their oral administration. Hence, proteins and peptides are mainly administered parenterally in aqueous solutions (Frokjaer Otzen 2005; Wang 1999). The most common type of physical instability is protein aggregation (Wang 1999; Wei Wang 2005). Aggregation occurs when a protein unfolds and the buried hydrophobic resides become exposed to the solvent. Hydrophobic parts of different protein molecules can then cluster together to form aggregates (Stefani 2004). Aggregation can occur during almost any of the biopharmaceutical manufacturing processes but especially during fermentation, refolding, purification, formulation and storage of the product, and the aggregation can either be reversible or irreversible. The aggregation can lead to no or reduced biological activity, it can reduce protein solubility and alter the protein immunogenicity. Therefore inhabitation of protein aggregation is of special importance. Methods for inhabitation of protein aggregation have been developed. One simple method is to include excipients in the protein formulation that inhibit the aggregation such as sugars, polyols, surfactants, salts, PEGs, polymers, metal ions, amino acids and cylcodextrins (Wang 1999; Wei Wang 2005). Cyclodextrins have been investigated and used to stabilize and inhibit aggregation of insulin by forming

inclusion complexes with lipophilic domains present in the side chain of insulin (Valentini et al. 2015). In this article we will investigate the use of cyclodextrins (CDs) in aqueous eye drops containing a protein drug, lysozyme, and a peptide drug, cyclosporin A (CyA). Previously we have studied the effects of CDs on the solubility and stability of CyA in aqueous solutions and in eye drops containing CyA nanoparticles (Johannsdottir et al. 2015).

1.2. Cyclodextrins (CDs)

CDs are cyclic oligosaccharides derived from starch containing six (αCD), seven (βCD), eight (γCD) or more (α-1,4)-glucopyranose units. They are cone shaped, due to the chair conformation of the glucopyranose units, with the primary hydroxyl groups of the sugar residues at the narrow edge of the cone and the secondary hydroxyl group at the wider edge (Fig. 1). The inside of the CD cone is lined with skeletal carbon and ethereal oxygen atoms which leads to a somewhat lipophilic character (Brewster and Loftsson 2007; Del

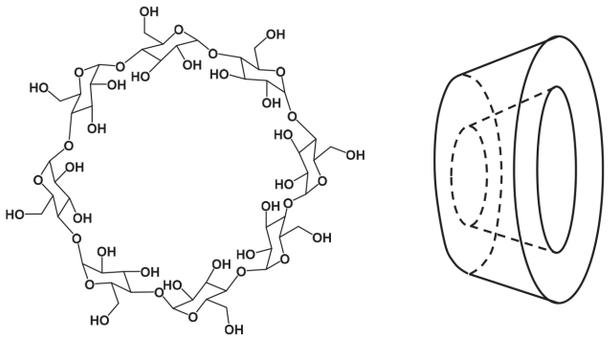

Fig. 1: The chemical structure and a schematic drawing of the γ-cyclodextrin (γCD) molecule. The molecule is cone shaped with the primary hydroxyl groups at the narrow edge of the cone and the secondary hydroxyl group at the wider edge.

Valle 2004; Loftsson and Brewster 1996; Loftsson and Brewster 2010; Uekama 1998). This results in a hydrophilic outer surface, where in aqueous solutions hydroxyl groups will form hydrogen bonds with surrounding water molecules, and a lipophilic central cavity, where small lipophilic molecules or lipophilic moieties of larger molecules can enter to form inclusion complexes (Brewster and Loftsson, 2007). Through formation of inclusion complexes CDs can both act as a solubilizers of poorly water-soluble drugs and increase drug stability by protecting sensitive molecular structures within the cavity. During complexation, no covalent bonds are formed or broken, and in aqueous solutions drugs located within the CD cavity are in a dynamic equilibrium with free drug molecules (Stella et al. 1999). In aqueous solutions the hydroxyl groups on the outer surface of CD molecules and CD/drug complexes can form hydrogen bonds with neighboring CD and drug molecules, as well as with other dissolved CD/drug complexes, and complexes thus formed are known as non-inclusion complexes. Formation of such non-inclusion complexes can lead to further self-assembling of CDs and their complexes with subsequent formation of nano-sized aggregates and micellar-like structures (Brewster and Loftsson 2007; Loftsson et al. 2002; Messner et al. 2011; Messner et al. 2010; Szejtli 1998).

1.3. Lysozyme

Lysozyme was discovered in 1922 by Alexander Fleming when he was investigating his own nasopharyngeal mucus. He discovered that lysozyme produced a weak antibacterial effect by breaking apart the bacteria cell wall (Tan and Tatsumura 2015). Later it was discovered that the antibacterial effect was due to the fact that lysozyme is a hydrolytic enzyme that cleaves the β-1,4 glycoside bond between *N*-acetylmuramic acid and *N*-acetylglucosamine in peptidoglycan, a carbohydrate found in bacterial cell walls (Kozuka et al. 2015; Swaminathan et al. 2011). Lysozyme can be found in human tears, saliva, skin, hair and fingernail as well as in hen egg white (HEW) (Tan and Tatsumura, 2015). HEW lysozyme is today one of the most investigated proteins in biochemistry. Its popularity is mainly due to the fact that HEW is rich of lysozyme that can easily be purified (Venkataramani 2013).

HEW lysozyme is a small (14.3 kDa) globular protein made of a single polypeptide chain comprising 129 amino acid residues with four intramolecular disulfide bridges (Rothwarf and Scheraga 1996; Swaminathan et al. 2011). It contains two structural domains forming the two halves of the active side cleft. One consists of four α-helixes along with a 3_{10} helix and the other domain contains a large triple-stranded antiparallel β-sheet, a 3_{10} helix and a long loop. Along with the disulfide bridge a short double-stranded antiparallel β-sheet also links these two domains (Matagne and Dobson 1998; Rothwarf and Scheraga 1996). Due to the four disulfide bridges, lysozyme is a relatively stable protein that generally does not aggregate in aqueous solutions (Sethuraman and Belfort 2005). This makes HEW lysozyme an ideal model protein for the development of new drug formulation technologies, such as aqueous eye drops containing cyclodextrin-carriers. The aqueous solubility of lysozyme has been reported to be 10 mg/ml in acetate buffer at pH 4.75 (Penkova et al. 2006).

1.4. Cyclosporine A (CyA)

CyA is a cyclic undecapeptide (i.e. oligopeptide containing 11 amino acids) with a molecular weight of 1206 Da. Seven of these amino acids are methylated, which gives CyA a lipophilic nature (Survase et al. 2011). It has an aqueous solubility of 0.008 mg/ml at ambient temperature and $LogP_{octanol/water}$ of 2.92 at 21 °C (Loftsson and Hreinsdottir 2006; Tayar et al. 1993). CyA is a potent drug with a wide variety of biology activities including immunosuppressant activity, anti-inflammatory and anti-fungal properties. It is one of the most commonly prescribed immunosuppressives to prevent rejection after organ transplants or to treat autoimmune diseases (Survase et al. 2011). It is also used for topical treatment of various ocular inflammatory diseases, such as dry eye disease and keratoconjunctivitis (Donnenfeld and Pflugfelder 2009). Previously we have described the development of 0.05% CyA eye drops, where we used the natural α-cyclodextrin (αCD) to solubilize the drug and the natural γ-cyclodextrin (γCD) to form CyA/CD complex aggregates (Johannsdottir et al. 2015). In this article we will compare CD interactions of the lipophilic oligopeptide CyA and the hydrophilic protein lysozyme.

2. Investigations, results and discussion

2.1. Complexation of cyclodextrins and lysosome or CyA

2.1.1. Solubility and buffer selection

The first step in the development of an aqueous drug formulation is to compose a medium that both solubilizes and stabilizes the drug. The solubility of lysozyme in water is good, and does not need any improvement. The aqueous solubility of CyA is, however, poor due to its lipophilicity. The effect of the three natural CDs (i.e. αCD, βCD, γCD) on the aqueous solubility of CyA was investigated, phase-solubility diagrams plotted, and CE was calculated from each slope (Table 1). Both βCD and γCD displayed A_L type diagrams, indicating that the CD/CyA complexes formed have 1:1 stoichiometry, but αCD has B_S indicating that the CyA/αCD complex formed has limited solubility in aqueous media (Higuchi and Connors 1965). αCD has a greater solubilizing effect on CyA and displays higher CE than the other two CDs. This indicates that CyA has a higher affinity to CDs with relatively small central cavity and, consequently, forms a complex with αCD more readily than with other CDs that have larger cavities. The increasing section of the CyA-αCD diagram has a positive deviation from linearity (i.e. is of A_P type) indicating that higher-order complexes with respect to αCD are most probably formed where more than one αCD molecule forms a complex with every one CyA molecule (e.g., 1:2 and 1:3 CyA/αCD complexes).

Table 1: The solubilizing effects of the three parent CDs on CyA in pure water (mean ± SD; n=3)

Cyclodextrin	Phase-solubility diagram	CE	Solubility (mg/ml) in the presence of 5% (w/v) CD	Solubility (mg/ml) in the presence of 15% (w/v) CD
αCD	B_S	0.54	0.76 ± 0.02	4.22 ± 0.59
βCD	A_L	0.030	βCD not soluble	βCD not soluble
γCD	A_L	0.0049	0.062 ± 0.001	0.11 ± 0.00

For lysozyme the first step is not to solubilize the protein, since it is fairly water-soluble, but rather to find an appropriated buffer system. Buffer is needed to maintain a constant pH and to obtain sufficient formulation stability (Zbacnik et al. 2017). The buffer salts also need to be able to coexist with CD in the solution without forming any types of aggregates. We tested three different buffers, HEPES (4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid), TRIS (TRIS(hydroxymethyl)aminomethan) and phosphate buffer with CD, and with and without lysozyme. When CD was dissolved in aqueous HEPES buffer, relatively large flake like aggregates were formed. Smaller aggregates were observed when the natural CDs were added to a phosphate buffer and virtually no aggregates were formed when the CDs were added to a TRIS buffer. Lysozyme did not have any effect on this aggregate formation. Thus, TRIS buffer was selected for further investigation.

2.1.2. Spectroscopic measurements

Visible spectroscopy measurements were performed to evaluate the cloudiness of the lysozyme solutions. The measurements showed that solutions containing αCD, HPβCD (2-hyroxypropyl-β-cyclodextrin), βCD and γCD were less transparent when lysozyme was present than when it was not (Table 2). The water soluble HPβCD had much less effect than the less soluble natural αCD, βCD and γCD. In general, HPβCD complexes have less tendency to aggregate and form nanoparticles than, for example, the natural γCD. This could be due to the fact that HPβCD consists of mixtures of a wide variety of geometrical isomers. This indicates that there is some aggregate formation in the samples containing lysozyme possibly due to aggregation of lysozyme/CD complexes although aggregation of pure lysozyme cannot be excluded.

Table 2: Absorbance of aqueous 20 mM TRIS buffers at pH 7.0, containing no lysozyme and containing 1.0 mg/ml lysozyme, at room temperature (approx. 22 °C)

	No CD	αCD	HPβCD		βCD		γCD
		10%	10 %	15%	0.6%	1.2%	5%
No lysozyme	-	0.22	0.009	0.031	0.09	0.02	0.05
Lysozyme	0.13	0.48	0.050	0.027	0.23	0.26	0.35

The CD concentrations are in % w/v.

2.1.3. Dynamic light scattering (DLS)

DLS shows that the size of the aggregates that are formed when CD and lysozyme are present together in the aqueous buffer solution varies from 1 nm to 1-2 μm and the results are rather similar for all CDs tested (Table 3). Similar results are obtained for the aqueous CyA formulation (Table 4). The DLS results indicate that aggregates are being formed but their size can vary depending on the type and concentration of CD. The aggregates are most likely unstable and constantly being formed and dissociated in aqueous solutions (Ryzhakov et al. 2016).

2.1.4. Solid drug fraction

The solid drug fraction for all tested solutions with lysozyme was found to be negligible. There was no detectable lysozyme in the solid fraction although particles as large as about 2 μm could be detected in the aqueous lysozyme formulations. This could indicate that the aggregates formed are kept together by relatively weak forces and disassociate upon centrifugation.

For CyA however, it was found that the solid drug fraction was 28.8 %, which means that 28.8 % of CyA in the formulation was found to be in the solid form that was separated from the solution upon centrifugation. Here the aggregates formed appear to be more stable than those containing lysozyme. This is due to the fact that CyA forms inclusion complexes with αCD, and those complexes form non-inclusion complexes with surrounding CD molecules and CD/CyA complexes. Lysozyme, with all its hydrophobic moieties located within this globular protein, is only able to form non-inclusion complexes with CDs.

Table 3: Dynamic light scattering results of solutions containing 1 mg/ml of lysozyme in an aqueous 20 mM TRIS buffer at pH 7.0 and 25 °C, containing various amounts of different CDs

Cyclodextrin	Size (nm)	Intensity %
10 % (w/v) αCD	1664	100
1.2 % (w/v) βCD	216.2	6.7
	609	20.1
	1376	44.4
	3520	28.5
0.6% βCD	0.99	2
	968	77.6
	2907	20.4
10 % (w/v) HPβCD	446	100
15 % (w/v) HPβCD	0.98	2.3
	3.54	6.6
	218.2	21.2
	820	70
5 % (w/v) γCD	1.04	2.6
	337	14.1
	788	56.9
	1294	26.4

Table 4: Dynamic light scattering results of solutions containing 0.5 mg/ml of CyA in an aqueous media containing 4% (w/v) αCD, 10% (w/v) γCD, 1.4% (w/v) polyvinyl alcohol, 0.02% (w/v) benzalkonium chloride and 0.1% (w/v) disodium edetate dehydrate

Size (nm)	Intensity %
1.10	4.10
5.14	29.5
244.3	64.2
731	2.26

2.2. Stability studies

CyA is a stable compound and is not folded like a protein, thus measurements of the physical stability were only made with lysozyme.

2.2.1. Stability of CDs in the presence of lysozyme

Since lysozyme cleaves glycoside bonds within bacteria cell walls and CD are formed by glucopyranose units linked together with glycoside bonds, studies were performed to see if lysozyme would also catalyze CD degradation in aqueous solutions. Aqueous 20 mM pH 7.0 TRIS buffer containing 1.8% (w/v) βCD and 1.0 mg/ml lysozyme was stored at room temperature for 11 days and the βCD concentration determined. The results showed that the βCD concentration was 98.7% of the initial concentration. No detectable αCD and γCD degradation was observed after storage of 10% (w/v) αCD and 5% (w/v) γCD in aqueous 20 mM pH 7.0 TRIS

buffer containing 1.0 mg/ml lysozyme at room temperature for up to 4 months. This indicates that lysozyme does not hydrolyze the parent CDs and negligible CD degradation is expected to occur during this study.

2.2.2. *Effect of guanidine lysozyme*

Guanidine hydrochloride (GuHCl) is a known denaturation agent that unfolds proteins. When GuHCl binds to the protein it induces conformational changes of lysozyme which can lead to unfolding of the protein, dependent on concentration (Hedoux et al., 2010). Our studies showed that lysozyme unfolds at GuHCl concentrations between 3.5 and 4.0 M (Fig. 2). The protein in buffer without CDs unfolds at slightly lower concentrations of GuHCl. This indicates that the CDs have a slightly stabilizing effect on the protein against chemical denaturation.

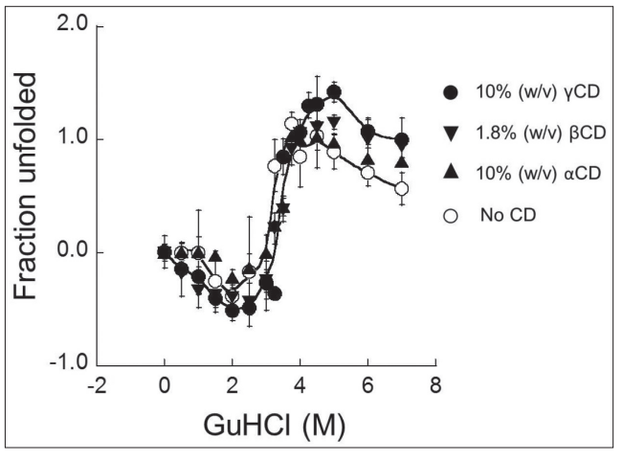

Fig. 2: The effect of guanidine hydrochloride (GuHCl) on the unfolding of lysozyme in aqueous 20 mM TRIS buffer solutions (pH 7.0; 25°C) containing different CDs (mean values ± standard deviation, n = 3). For clarification solid curves are drawn through the data obtained in just pure medium and the medium containing 10% (w/v) γCD.

2.2.3. *Thermal stability*

Thermal unfolding is linear with increased temperature (Fig. 3). The CDs seems to have negligible effects on the temperature denaturation of lysozyme. Only γCD has slightly protecting properties, and, if anything, βCD appears to increase the unfolding of lysozyme.

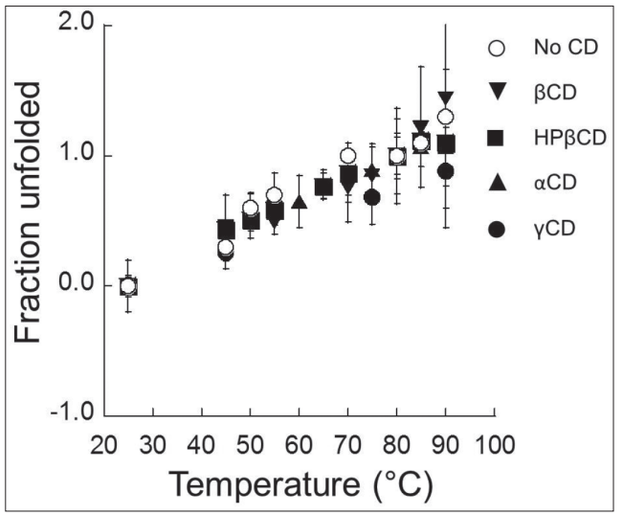

Fig. 3: Thermal unfolding of lysozyme in aqueous 20 mM TRIS buffer solutions (pH 7.0) containing different CDs (mean values ± standard deviation, n = 3).

2.2.4. *Recovery of activity after a heat shock*

The highest lysozyme recovery was obtained from the blank (Fig. 4). The recovery was lower from the CD solutions. When proteins are folded their hydrophobic parts are buried inside the protein, when the protein unfolds, however these hydrophobic groups open up to the surface. CDs can form inclusion complexes with these groups that can delay or inhibit refolding of the protein hence their recovery of the activity. γCD has a greater ability to form inclusion complexes with the unfolded lysozyme resulting in only 9% recovery. Apparently, the hydrophobic groups exposed by unfolding of the globular lysozyme molecule fit better into the larger γCD cavity than into the smaller αCD and βCD cavities.

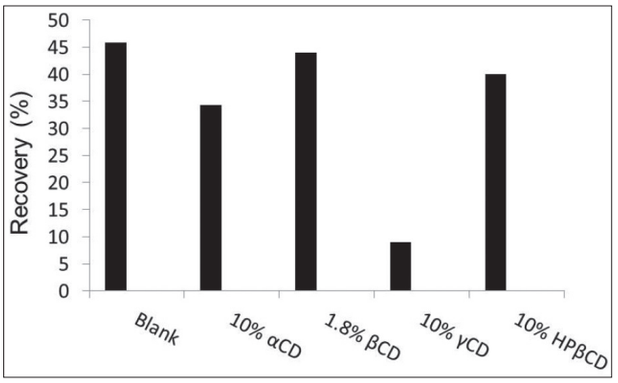

Fig. 4: Recovery of lysozyme activity after a heat shock in different CD solutions (% w/v).

2.3. Conclusions

CyA is a lipophilic oligopeptide (molecular weight 1.2 kDa) that consists of only 11 amino acids, 7 of which are methylated. It has very limited solubility in water of only 0.008 mg/ml at ambient temperature. This peptide behaves similar to low molecular weight drugs with similar physicochemical properties such as steroids. Thus, in aqueous CD solutions, CyA forms inclusion complexes where lipophilic structures of the CyA molecule enter the central cavity of the CD molecules. Then the complexes formed can self-assemble to form aggregates. The forces keeping the aggregates together are relatively weak and the aggregates dissociate upon media dilution. However, the aggregates can be centrifuged from the aqueous media. Aqueous CyA eye drops containing CyA/CD complex aggregates have been prepared and are currently being tested in rabbits (Johannsdottir et al. 2015).

HEW lysozyme is a small (14.3 kDa) globular protein made of a single polypeptide chain comprising 129 amino acid residues. The polypeptide chain is folded to form a globular structure where the non-polar amino acids are buried within the protein interior while the exterior is hydrophilic. Thus, lysozyme is water-soluble (solubility about 10 mg/ml) and unable to form inclusion complexes with CDs. Lysozyme is able to form non-inclusion complexes with CDs and those complexes can self-assemble to form aggregates. The lysozyme/CD complex aggregates are kept together by very weak forces and dissociate upon media dilution and centrifugation. The lysozyme/CD non-inclusion complexes can offer some protection against chemical and thermal denaturation. However, γCD forms complexes with the unfolded lysozyme molecules and hampers refolding of the molecules after heat shock. This makes CDs less suitable as enabling excipients in aqueous eye drop formulations containing lysozyme.

4. Experimental

4.1. Materials

Lysozyme from chicken egg white, lysozyme assay kit, guanidine hydrochloride 92%, sodium chloride, 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid (HEPES), sodium phosphate dibasic, sodium phosphate monobasic, methanol HPLC grade (≥99.9%; MeOH), acetonitrile HPLC grade (≥99.93%; ACN), poly(vinyl alcohol)

87-91% hydrolizes (average MW 30-72 kDa) and benzalkonium chloride were purchase from Sigma Aldrich (St. Louis, Mo, USA), α-cyclodextrin (αCD), β-cyclodextrin (βCD), γ-cyclodextrin (γCD), 2-hydroxypropyl-β -cyclodextrin (HPβCD) with molar substitution (MS 0.6-0.9) (MW 1380 – 1500 Da) were purchased from Wacker Chemie (Munich, Germany), TRIS(hydroxymethyl)aminomethan (TRIS) and disodium edetate dehydrate (EDTA) were purchase from Merck (Darmstadt, Germany). Milli-Q (Millipore, Bierica, MA) water was used for the preparation of all solutions.

4.2. Solubility and buffer selection

The solubility of CyA was determined by the heating method using sonication (Loftsson and Hreinsdottir 2006; Loftsson et al. 2005). An excess amount of drug was added to aqueous solutions containing up to 20% (w/v) CDs. The solutions were sonicated at 40-50 °C for 45-60 min in sealed glass vials, and then allowed to cool to room temperature (22-23 °C). A small amount of solid drug was then added to each vial, the vial was resealed and allowed to equilibrate under constant agitation and protected from light for 7 days at room temperature. When the solutions had reached equilibrium, they were filtered through a 0.45 µm membrane filter and analyzed by HPLC. The apparent complexation constant for CyA/CD complexes ($K_{1:1}$) was determined using the phase-solubility method developed by Higuchi and Connors (1965). The complexation efficiency (CE) was determined from the slope of phase-solubility diagrams (plots of total solubility of the drug versus total CD concentration in mol/l):

$$CE = \frac{slope}{1 - slope} = K_{1:1} \cdot S_0$$

where S_0 is the intrinsic solubility of the drug.
To find a suitable buffer systems for lysozyme to mix with CDs at high concentrations, three different buffers were selected and tested. These buffers were 20 mM HEPES, TRIS and sodium phosphate buffers, all of them where adjusted to pH 7. 5, 10 and 15% (w/v) of αCD, γCD and HPβCD, as well as 0.6 and 1.2% (w/v) βCD were mixed separately to each buffer solution with and without 1 mg/ml lysozyme. These mixtures were then shaken for 5 days and the appearance of the mixture was visually observed.

4.3. Solid drug fraction

For lysozyme: 1 mg/ml lysozyme was mixed with 10 and 5 % (w/v) of αCD, γCD and HPβCD as well as 0.6 and 1.2% (w/v) of βCD in 20mM TRIS buffer at pH 7 with and without 150 mM of NaCl and shaken for 5 days. One ml of each solution was centrifuged at 13.000 rpm (Heraeus Pico 17 Centrifuge, Thermo Fishger Scientific) at room temperature (22-23 °C) for 60 min and the quantities of lysozyme in the supernatant analyzed by HPLC.
For CyA: CyA eye drop formulation, which contained 4% (w/v) αCD, 10% (w/v) γCD, 0.05% CyA (w/v), 1.4% (w/v) PvA. 0.02% (w/v) benzalkonium chloride and 0.1% (w/v) EDTA, was shaken for a week, then centrifuged at 6000 rpm (MC6 centrifuge, Sarstedt AG, Nümbrecht, Germany) at room temperature (22-23 °C) for 20-30 min and the quantities of CyA in the supernatant analyzed by HPLC
The drug content for both lysozyme and CyA in solid phase was calculated as:

$$\%\,solid\ drug\ fraction = \frac{(Total\ drug - dissolved\ drug)}{Total\ drug\ content} \times 100$$

4.4. Dynamic light scattering

The particle size characterization of the eye drop formulations was performed by dynamic light scattering (DLS), using a Nanotrac Wave particle size analyzer from Microtrac Inc. (Montogomeryville, USA). Measurements were carried out at 25 °C, 180° scattering angel and a 780 nm laser beam, and each measurement was done in triplicate.

4.5 Spectroscopic measurements

Visible spectroscopy measurements were performed on a Multiskan ascent microplate from Thermo Fisher (Wallham, Ma, USA) and the absorbance was measured at 450 nm.

4.6. Quantitative analysis

The quantitative analysis of lysozyme was performed on a high-performance liquid chromatography (HPLC) component system Ultimate 3000 Series from Dionex Softron GmbH (Germering, Germany) consisting of a LPG-3400A pump, SR-3000 solvent rack, auto sampler column compartment, VWD-3400 UV/Vis detector and Waters Xbridge BEH125 size exclusion 7.8*150 mm, 3.5-micron column. The mobile phase consisted of 0.1 M sodium phosphate buffer and 0.3 M NaCl, the flow rate was 0.86 ml/min and the detection wavelength was 214 nm. The column was calibrated with BEH 125 waters standard mix, that consisted of 4 component protein mixture (Thyroglobulin, Ovalbumin, Ribonuclease A and Uracil). The assay method was validated.
The quantitative analysis of CyA was performed on a reversed-phase high-performance liquid chromatography (HPLC) component system Ultimate 3000 Series from Dionex Softron GmbH (Germering, Germany) consisting of a DGP-3600A pump, SRD-3600 solvent rack and degasser, WPS-3000TLS well plate sampler, TCC-3100 column compartment, photodiode array detector and Phenomenex Luna C-18 150 mm x 4.60 mm and 5 micron column, with a matching guard column. The mobile phase consisted of acetonitrile, methanol and water (60:20:20), the flow rate was 1 ml/min, column oven temperature was 80 °C and the detection wavelength was 205 nm

4.7. Quantitative analysis of cyclodextrins

1.8% (w/v) βCD in 20 mM TRIS buffer pH 7 with and without 1 mg/ml protein was allowed to stand for 11 days. Then the concentration of βCD was measured and compared. 5 % (w/v) ϒCD and 10% (w/v) αCD in 20 mM TRIS buffer pH 7 with and without 1 mg/ml lysozyme were stored at room temperature for 7 days and at 5 °C for 4 months. Concentration of CD was measured and compared.
Quantitative analysis were performed on a reverse-phase high performance liquid chromatography (HPLC) system (Dionex Softron GmbH,Germany), Ultimate 3000 series, consisting of a LPG-3400SD pump with a built-in degasser, a WPS-3000 autosampler, a TCC-3100 column compartment and a Corona ultra RS detector. Phenomenex Kinetex C18 150 mm × 4.60 mm, 5 µm column with SecurityGuard ULTRA Cartridges UHPLC C18 (Phenomenex, UK) were used as stationary phase. For quantitative analysis on βCD a gradient mobile phase was as follows: 0-4 min 5% ACN 95 % water, 4-6 min from 5% to 30 % ACN, 6-11 min 30% ACN 70% water, 11-13 min 30% to 5% ACN and 13-15 min 5% ACN and 95% water with the flow rate 1 mg/min. The temperatures of the column, sampler compartments and nebulizer of detector were set at 30, 25 and 30 °C, respectively. Chromatograms were evaluated using Chromeleon version 6.80. The injection volume was 50 µl. For quantitative analysis on γCD and αCD the mobile phase was 10% MeOH and 90% water with flow rate 1.0 ml/min. The temperatures of the column, sampler compartments and nebulizer of detector were set at 30, 25 and 30 °C, respectively. Chromatograms were evaluated using Chromeleon version 6.80. The injection volume was 20 µl.

4.8. Fluorescence Spectroscopy

Both temperature and chemical stability were measured with a Perking Elmer LS 55 Fluorescence spectrometer (Massachusetts, USA) with excitation 286 nm and emission absorbed at 280-460 nm
Effect of guanidine: 1 mg/ml lysozyme in 20mM TRIS buffer at pH 7 with and without 10% (w/v) αCD, γCD and HPβCD and 1.8% (w/v) βCD were measured with different GuHCl concentrations, ranging from 0-7 M. Each concentration was measured in triplicate. Fractions of the unfolded protein (fu) were found by the following equation:

$$fu = \frac{(S0 - Sf)}{(Su - Sf)}$$

where S represents the measured intensity at 358 nm, S_0 is the intensity of the tested protein, S_f is the intensity when the protein is completely folded, and Su is the intensity of completely unfolded protein.
Temperature stability: 1 mg/ml lysozyme in 20 mM TRIS buffer at pH 7 with and without 10% (w/v) αCD, γCD and HPβCD and 1.8% (w/v) βCD were heated up and measured at different temperature points in triplicate. Temperature points ranged from 25-85 °C, after reaching each temperature point the solution was allowed to reach equilibrium for 10 min before measurements were taken. Fractions of the unfolded protein (fu) were found by equation 3, where S represents the measured intensity at 345 nm.

4.9. Activity measurements

Lysozyme activity was measured using lysozyme activity kit from Sigma Aldrich (St. louis, Mo, USA). Briefly, 12 µl of sample for testing were added to one well in a 96 well plate. Than 320 µl of Micrococcus lysodeikticus cell suspension, which was prepared in 20 mM TRIS buffer at pH 7.4, was added to the well and shaken for 10 s. Then the decrease in absorbance at 450 nm wavelength was monitored for 5 min in a Multiskan ascent microplate reader from Thermo Fisher (Waltham, Ma, USA).
Recovery of activity after a heat shock: 0.01 mg/ml lysozyme in 20 mM in TRIS buffer at pH 7 with and without 10% (w/v) αCD, γCD, HPβCD or 1.8% (w/v) βCD were heated to 75 °C and kept at that temperature for 10 min. Then the solutions were allowed to cool down to room temperature and stand for 30 min. The activity of these solutions was measured as well as the activity of solutions that had not been treated with a heat shock. Recovery of heat shocked proteins was found by comparing the activity of heat shocked protein with the unheated protein.

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