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Pullulan and trehalose based films for protein delivery in the oral cavity

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INTRODUCTION

Orodispersible films (ODFs) are single- or multilayer sheets of suitable materials, to be placed in the mouth where they disperse rapidly [1]. So, they do not need to be swallowed and can be administered without water, which makes them excellent candidates for some uncooperative users, like children and psychiatric patients. Also, ODFs with fast dissolution rate, high durability and good patient compliance are promising carriers for proteinaceous drug substances.

However, during the preparation of protein loaded ODFs, the protein is subjected to various stresses (e.g. shear stress), which can deteriorate the protein. It is well known that sugars can be used for the stabilization of proteins [2]. Trehalose, a disaccharide, is widely accepted as the gold standard for protein stabilization, because it can form a tight coating around the protein. However, small sized sugars have poor film forming properties. On the other hand, pullulan, a polysaccharide, has excellent film forming properties [3], but is not able to form a compact coating around the protein. Therefore, we hypothesized that a combination of trehalose and pullulan would be ideal for the preparation of a protein containing ODF [4]. In this study we investigated the applicability of these combinations in orodispersible films for the stabilization of two model proteins, a rather stable protein, i.e. lysozyme and a highly unstable protein, i.e. β -galactosidase.

MATERIALS AND METHODS

Materials

Pullulan (average molecular weight 200-300 kDa) and trehalose were kind gifts from Hayashibara (Okayama, Japan). Lysozyme (from chicken egg white, Mw = 14 kDa) and micrococcus lysodeikticus were obtained from Sigma-Aldrich (St. Louis, USA). β -galactosidase (from Escherichia Coli, Mw = 540 kDa) was purchased from Sorachim (Lausanne, Switzerland). Glycerol 85% and Tween 80 were obtained from Bufa (IJsselstein, The Netherlands). All other excipients and chemicals were of analytical grade.

Preparation of the Casting Solution And ODFs

Different casting solutions were prepared as listed in Table 1. All excipients were added to water under constant stirring. After the excipients were completely dissolved, the

solution was stirred at low speed (100 rpm) to obtain a clear and homogeneous solution. After that, lysozyme or β -galactosidase (0.4 % for the total amount of trehalose and pullulan) was added and stirred at low speed for an additional 4 hours.

ODFs were prepared using the solvent casting method as published before [5]. The casting speed was 10 mm/s with a casting height of 1000 μ m. Subsequently, the film layers were dried for 48 h at 30 °C and a relative humidity (RH) of 60-80 % or freeze-dried for 48 h (at temperature -35°C and a pressure of 0.220 mbar for 24 h and decreasing pressure to 0.050 mbar and increasing temperature to 25 °C for 24 h). ODFs were punched using an Artemio perforator (Artemio, Wavre, Belgium) and scissor in rectangles of 1.8 \times 0.9 cm, which were put into eppendorf tubes for stability studies.

Samples	Trehalose (g)	Pullulan (g)	Glycerol (g)	Tween 80 (g)	Protein (mg)	Water (up to) (g)
0/100 films	0	1.500	0.265	0.058	6	10
20/80 films	0.375	1.500	0.331	0.058	7.5	10
30/70 films	0.643	1.500	0.378	0.058	8.6	10
40/60 films	1.000	1.500	0.441	0.058	10	10

Table 1. Composition of different casting solutions.

Biological Activity of Lysozyme

The biological activity of lysozyme was measured with a turbid metric assay. Lysozyme causes lysis of bacteria, which leads to a decrease in the turbidity of a bacterial suspension. Therefore, the activity of lysozyme can be measured from the rate of turbidity decrease of Micrococcus Lysodeikticus. Briefly, ODFs (at the size of 1.8 \times 0.9 cm) in eppendorf tubes were dissolved with 1.0 mL of 0.1 M phosphate buffer (pH 7.3) and at the same time serial dilutions with concentration ranging from 0-125 μ g/mL of lysozyme in phosphate buffer (pH 7.3) were prepared as calibration curve. The bacterial Micrococcus Lysodeikticus was suspended in 66 mM phosphate buffer pH 6.2 at a concentration of 2.5 mg/mL. Further, 20 μ L

samples in triplicate was pipetted into the wells of 96-well plates. After 200 μL of bacterial suspension was pipetted into each sample, the absorbance was measured at 415 nm during 4 min with an interval of 18 sec. The activity of lysozyme was calculated from slope of test samples and lysozyme activity was corrected with the weight of ODFs.

Enzymatic Activity of β -galactosidase

The enzymatic activity of β -galactosidase (BG) was determined using a kinetic enzymatic assay, based on the rate of conversion of a colorless substrate, o-nitrophenyl- β -galactoside, into the yellow product, o-nitrophenol, by BG. First, ODFs in eppendorf tubes were dissolved in 1.0 mL of 0.1 M phosphate buffer (pH 7.3) and diluted 10 times in 50 mM phosphate buffer supplemented with 0.1% BSA and 1 mM MgCl_2 . Subsequently, 20 μL sample was pipetted into each well of a 96-well microplate (Greiner Bio-One, F shape), followed by 200 μL MgCl_2 solution (1.4 mM MgCl_2 in 0.1 M phosphate buffer, pH 7.3). The plate was incubated at 37 $^\circ\text{C}$ for 10 min. Afterwards, 20 μL of 50 mM o-nitrophenyl- β -galactoside was pipetted into the wells and the absorption was measured at 405 nm for 15 min at 37 $^\circ\text{C}$ with an interval of 30 s (Synergy HT Microplate Reader, BioTek Instruments, Winooski, VT). The activity of BG in ODFs was calculated from the slope of this conversion. The results were statistically analyzed using two-way analysis of variance (ANOVA). A p-value <0.05 was considered as significantly different.

RESULTS AND DISCUSSION

Lysozyme Stability

The process stability of lysozyme for both air-drying and freeze-drying was around 100 %. After stored at 30 $^\circ\text{C}$, 0% RH for 4 weeks, there was no significant decrease in activity of lysozyme incorporated in either air-dried or freeze-dried ODFs.

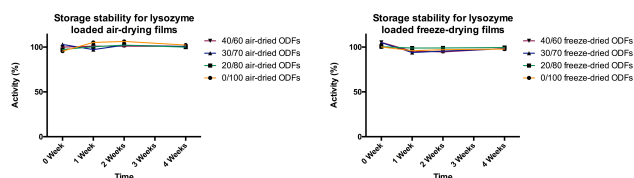


Figure 1. Process and storage stability of lysozyme loaded ODFs.

β -galactosidase Stability

After preparation, the air-dried ODFs clearly showed a trend towards increased stability of BG with an increased trehalose/pullulan ratio, indicating that with the addition of trehalose to pullulan a remarkable stabilizing effect on β -galactosidase can be achieved. After 1 and 2 weeks of storage of these ODFs at 30 $^\circ\text{C}$, 0% RH, the same trend remained in spite of some decrease of BG activity. At all

trehalose/pullulan ratios, BG was better stabilized during preparation by freeze-drying than by air-drying. In addition, the freeze-dried ODFs also showed a trend towards increased stability of BG with an increased trehalose/pullulan ratio, although less pronounced. After 1 and 2 weeks of storage at 30 $^\circ\text{C}$, 0% RH, however, the activity of BG was dramatically decreased, in particular for the 0/100, 20/80 and 30/70 formulation.

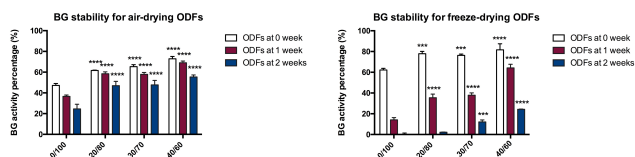


Figure 2. Process and storage stability of β -galactosidase loaded ODFs (** $P \leq 0.001$; *** $P \leq 0.0001$ compared to 0/100 formulations).

CONCLUSION

Lysozyme could be incorporated in ODFs without loss of activity and remained stable for at least 4 weeks at 30 $^\circ\text{C}$, 0% RH, independent on the production method or trehalose/pullulan ratio. BG, however, showed a clear trend towards increased stability when the trehalose/pullulan ratio was increased. Furthermore, BG incorporated in ODFs by freeze-drying showed a better process stability, while air-drying showed a better storage stability.

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