

University of Groningen

Addition of Pullulan to Trehalose Glasses Improves the Stability of β -Galactosidase at High Moisture Conditions

Teekamp, Naomi; Tian, Yu; Visser, J. Carolina; Olinga, Peter; Frijlink, Henderik W.; Woerdenbag, Herman J.; Hinrichs, Wouter L. J.

Published in:
Carbohydrate Polymers

DOI:
[10.1016/j.carbpol.2017.08.084](https://doi.org/10.1016/j.carbpol.2017.08.084)

IMPORTANT NOTE: You are advised to consult the publisher's version (publisher's PDF) if you wish to cite from it. Please check the document version below.

Document Version
Publisher's PDF, also known as Version of record

Publication date:
2017

[Link to publication in University of Groningen/UMCG research database](#)

Citation for published version (APA):

Teekamp, N., Tian, Y., Visser, J. C., Olinga, P., Frijlink, H. W., Woerdenbag, H. J., & Hinrichs, W. L. J. (2017). Addition of Pullulan to Trehalose Glasses Improves the Stability of β -Galactosidase at High Moisture Conditions. *Carbohydrate Polymers*, 176, 374-380. <https://doi.org/10.1016/j.carbpol.2017.08.084>

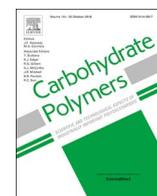
Copyright

Other than for strictly personal use, it is not permitted to download or to forward/distribute the text or part of it without the consent of the author(s) and/or copyright holder(s), unless the work is under an open content license (like Creative Commons).

Take-down policy

If you believe that this document breaches copyright please contact us providing details, and we will remove access to the work immediately and investigate your claim.

Downloaded from the University of Groningen/UMCG research database (Pure): <http://www.rug.nl/research/portal>. For technical reasons the number of authors shown on this cover page is limited to 10 maximum.



Addition of Pullulan to Trehalose Glasses Improves the Stability of β -Galactosidase at High Moisture Conditions



Naomi Teekamp, Yu Tian, J. Carolina Visser, Peter Olinga, Henderik W. Frijlink, Herman J. Woerdenbag, Wouter L.J. Hinrichs*

Department of Pharmaceutical Technology and Biopharmacy, University of Groningen, A. Deusinglaan 1, 9713 AV Groningen, The Netherlands,

ARTICLE INFO

Keywords:

pullulan
trehalose
protein stabilization
sugar glass
dynamic vapor sorption
differential scanning calorimetry

ABSTRACT

Incorporation of therapeutic proteins in a matrix of sugar glass is known to enhance protein stability, yet protection is often lost when exposed to high relative humidity (RH). We hypothesized that especially in these conditions the use of binary glasses of a polysaccharide and disaccharide might yield advantages for protein stability. Therefore, different amounts of the polysaccharide pullulan were introduced in freeze-dried trehalose glasses. In these homogeneous blends, the presence of pullulan above 50 weight % prevented crystallization of trehalose when exposed to high RH. Storage stability testing up to 4 weeks of the model protein β -galactosidase incorporated in pullulan/trehalose blends showed superior behavior of pure trehalose at 30 °C/0% RH, while pullulan/trehalose blends yielded the best stability at 30 °C/56% RH. In conclusion, binary glasses of pullulan and trehalose may provide excellent stability of proteins under storage conditions that may occur in practice, namely high temperature and high RH.

1. Introduction

Proteins are becoming an increasingly relevant class of drugs in today's pharmacotherapy. Due to their size as well as their complex and labile structure, ensuring stability of therapeutic proteins requires a completely different formulation approach than for conventional small molecule drugs (Manning, Chou, Murphy, Payne, & Katayama, 2010; Mitragotri, Burke, & Langer, 2014). Generally, pharmaceutical proteins are formulated as aqueous solutions, which have limited shelf life and often require refrigerated storage and transportation. Next to the costs and inconvenience involved with this so-called cold chain (Saxenian, 2017), proteins are often still subject to degradation. In case of impaired stability of proteins, not only their efficacy may be reduced, but degradation products may also elicit undesired immune reactions, with serious consequences for the patient (Jiskoot et al., 2012; Ratanji et al., 2014). Conversely, by storing proteins in the dry state, the degradation rate may be drastically reduced due to limited molecular mobility (Lai & Topp, 1999). However, the stresses during drying and subsequent storage could still impair their integrity (Mensink, Frijlink, van der Voort Maarschalk, & Hinrichs, 2017). Therefore, appropriate protection of the protein against such stresses, during both manufacturing and storage, is necessary.

Sugars are widely used for the stabilization of proteins during drying and subsequent storage. During the drying process, sugars enclose proteins in a glassy matrix yielding a so-called sugar glass. In literature, there are two leading theories on the mechanism of stabilization of sugar glasses: the vitrification and water replacement theory (Cicerone, Pikal, & Qian, 2015; Mensink et al., 2017). Upon vitrification in a glassy matrix, the molecular mobility of proteins is strongly reduced, which in turn reduces degradation reaction rates (Chang et al., 2005; Crowe et al., 1998). Next to this kinetic stabilization mechanism, the water replacement theory has been proposed. This theory describes protein stabilization based on thermodynamics. According to the water replacement theory, the hydrogen bonds between the water molecules in the hydration shell of the protein and the protein itself are (partially) replaced by hydrogen bonds with the sugar (Allison, Chang, Randolph, & Carpenter, 1999; Mensink et al., 2015). This replacement conserves the three-dimensional structure of the protein. It is presumed that these two mechanisms are not exclusive and they simultaneously contribute to the stabilization of proteins (Crowe et al., 1998; Mensink et al., 2017). For optimal stabilization according to both theories, a close contact of the sugar with the protein and a high physical stability of the carbohydrate matrix, *i.e.* a high glass transition temperature (T_g), are required.

Abbreviations: BSA, bovine serum albumin; DSC, differential scanning calorimetry; DVS, dynamic vapor sorption; HEPES, 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid; RH, relative humidity; T_g , glass transition temperature; T_g' , glass transition temperature of the maximally freeze concentrated fraction

* Corresponding author.

E-mail address: w.l.j.hinrichs@rug.nl (W.L.J. Hinrichs).

<http://dx.doi.org/10.1016/j.carbpol.2017.08.084>

Received 6 June 2017; Received in revised form 15 August 2017; Accepted 18 August 2017

Available online 24 August 2017

0144-8617/ © 2017 The Author(s). Published by Elsevier Ltd. This is an open access article under the CC BY license (<http://creativecommons.org/licenses/by/4.0/>).

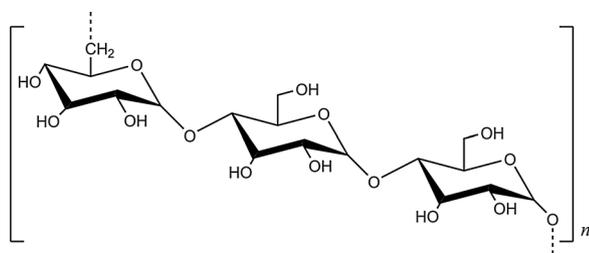


Fig. 1. Chemical structure of pullulan.

Numerous types of sugars have been explored as protein stabilizers (Chang et al., 2005; Hinrichs, Prinsen, & Frijlink, 2001; Tonnis et al., 2015). For various reasons outlined below, we hypothesize that the polysaccharide pullulan could be an attractive alternative. Pullulan is a linear and neutral exopolysaccharide produced by the yeast *Aureobasidium pullulans* consisting of repeating α -1,4 linked maltotriose units, which are connected by α -1,6 linkages (Fig. 1). Due to limited molecular flexibility arising from the latter connection, the conformation is believed to be random-coiled (Shingel, 2004). The molecular weight of the chains can vary between 45 and 2000 kDa (Cheng, Demirci, & Catchmark, 2011).

Over the last decades, pullulan has found various applications in the food and pharmaceutical industries. It is a tasteless, odorless, non-toxic and edible powder, and is generally regarded as safe. Pullulan readily dissolves in water and the resulting solutions are not affected by heat and pH changes (Prajapati, Jani, & Khanda, 2013). Therefore, pullulan solutions have for example been applied as low-viscosity fillers in blood plasma substitute solutions (Cheng et al., 2011; Prajapati et al., 2013). Furthermore, pullulan and pullulan derivatives have been applied in various drug delivery systems such as orodispersible films (Garsuch, 2009; Vuddanda et al., 2017), nanoparticles for protein delivery (Dionisio et al., 2013), and targeted delivery systems to the liver (Xi et al., 1996).

Although pullulan has many potential applications in drug delivery, it has never been thoroughly investigated as a sugar glass for protein stabilization. Yet, pullulan's high T_g and the absence of reducing groups would make it potentially an excellent candidate for protein stabilization. In fact, pullulan has been applied to enhance the stability of protein assay components by casting tablets in an exploratory study (Jahanshahi-Anbuhi et al., 2016). However, despite their high T_g , polysaccharides often show less efficient protein stabilization than the stabilization acquired by disaccharides, which have lower T_g s. Tonnis et al. (2015) hypothesized that polysaccharides are too bulky to form a compact coating around the protein. Furthermore, they also found that the addition of a disaccharide to a polysaccharide can improve the storage stability. This way, both a close contact between sugar and protein and a high physical stability are ensured.

Considering the above, we hypothesize that the addition of pullulan with its exceptionally high T_g to disaccharide glasses enhances the protein stabilizing properties, particularly at high humidity. Trehalose was selected as disaccharide as it has been well recognized as the gold standard for protein stabilization (Balcão & Vila, 2014; Manning et al., 2010). Firstly, physicochemical properties of pullulan and blends with a wide range of pullulan/trehalose ratios were assessed, including their behavior at conditions up to 90% relative humidity. Secondly, the stability of a model protein (β -galactosidase) in pullulan and pullulan/trehalose blends was tested at different temperature and humidity conditions. The applied high humidity conditions were chosen to demonstrate the specific beneficial effects of polysaccharides, as opposed to the low relative humidity conditions that are usually applied in storage stability studies with disaccharide stabilized proteins.

2. Materials and methods

2.1. Materials

Pullulan (average molecular weight 200–300 kDa, < 10% mono-, di- and oligosaccharides) was a kind gift of Hayashibara (Okayama, Japan). Trehalose was obtained from Cargill (Amsterdam, The Netherlands). β -galactosidase was purchased from Sorchim (Lausanne, Switzerland). Bovine serum albumin (BSA), disodium hydrogen phosphate dodecahydrate, 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid (HEPES), lithium chloride, magnesium chloride hexahydrate, *ortho*-nitrophenyl- β -galactoside, monosodium phosphate dehydrate and potassium acetate were purchased from Sigma Aldrich (Zwijndrecht, The Netherlands). Ammonium chloride was obtained from Spruyt-Hillen (IJsselstein, The Netherlands). Barium chloride was obtained from Merck (Darmstadt, Germany). Sodium bromide was purchased from Fagron (Capelle aan den IJssel, The Netherlands). Sodium nitrite was purchased from Boom (Meppel, The Netherlands). Potassium carbonate was obtained from Acros Organics (Geel, Belgium).

All solutions were prepared using Millipore water. The 0.1 M phosphate buffer used in this study consisted of 75 mM disodium hydrogen phosphate dodecahydrate and 25 mM monosodium phosphate dihydrate; pH was adjusted to 7.3 using 1 M NaOH. The pH of 2 mM HEPES buffer used in this study was set to 7.4.

2.2. Preparation of powder formulations

Pullulan and trehalose were accurately weighed and dissolved separately in water (dynamic vapor sorption and differential scanning calorimetry measurements) or HEPES buffer (all other samples). Next, the solutions were mixed in the following weight ratios of pullulan/trehalose: 1/0; 5/1; 2/1; 1/1; 1/2; 1/5 and 0/1 (final sample concentration of 50 mg/mL). For the storage stability experiments, β -galactosidase dissolved in HEPES buffer was added to each combination in a 1:249 protein:sugar weight ratio (final sample concentration of 50 mg/mL). As a negative control, a solution of β -galactosidase in HEPES buffer without any sugar was used. The closed vial storage stability samples were prepared in 4 mL vials, type I glass, Fiolax clear (Schott, Müllheim, Germany), with corresponding rubber stoppers (Salm & Kipp, Breukelen, The Netherlands). All other samples were prepared in 2 mL polypropylene vials. The vials were then immersed in liquid nitrogen to freeze the solutions and placed in a Christ-Epsilon 2-4 freeze-dryer (Salm & Kipp, Breukelen, The Netherlands) on a precooled shelf (-50°C). Next, the samples were freeze-dried at a shelf temperature of -35°C and a pressure of 0.220 mbar for 24 h. Secondary drying was performed by decreasing pressure to 0.050 mbar and increasing shelf temperature to 25°C during 24 h. After freeze-drying, the 4 mL vials were closed under dry nitrogen gas to ensure 0% RH. Samples were stored at -20°C until further analysis or subjection to a storage stability study.

2.3. Dynamic Vapor Sorption (DVS)

The water sorption isotherms of freeze-dried samples of all pullulan/trehalose compositions were determined using a DVS-1000 gravimetric sorption analyzer (Surface Measurement Systems Limited, London, UK). Samples had an initial mass of about 10 mg and were analyzed at ambient pressure and 25°C . The water uptake by the sugar blends was measured from 0% to 90% RH in 10% increments. The RH was increased after equilibrium was achieved (change in mass < 0.5 μg within 10 min).

2.4. Differential Scanning Calorimetry (DSC)

The glass transition and crystallization behavior of freeze-dried pullulan/trehalose blends was assessed using DSC. The T_g of the maximally freeze concentrated fraction (T_g') of pullulan/trehalose solutions was analyzed using a DSC 2920 (TA Instruments, Ghent, Belgium). First, 40–50 mg of aqueous solution (45 mg/mL) was accurately weighed in open aluminum pans. Samples were analyzed without lid. After placing in the DSC, the solution was frozen to -50 °C. Next, the temperature was increased to 20 °C at a rate of 20 °C/min. The inflection point in the heat flow versus temperature curve was taken as the T_g' .

The T_g' values for all blends were fitted using the Gordon-Taylor equation:

$$T_{g,mix} \approx \frac{\omega_1 \cdot T_{g,1} + k \cdot \omega_2 \cdot T_{g,2}}{\omega_1 + k \cdot \omega_2} \quad (1)$$

where ω_i is the mass fraction of component i , $T_{g,i}$ the glass transition temperature of component i and the constant k is a fitting parameter. In our calculations, trehalose was taken as component 1 and pullulan as component 2. To calculate the fitting parameter k , the water content was considered as constant and was not incorporated in Eq. (1).

The T_g of anhydrous pullulan/trehalose blends was analyzed using a Q2000 DSC (TA Instruments, Ghent, Belgium). An amount of 2 to 5 mg of freeze-dried material was accurately weighed in open aluminum pans. Samples were analyzed without lid. To remove residual water, the samples were preheated at 70 °C for 5 min. After equilibration at 20 °C, the temperature was raised to 300 °C at a rate of 20 °C/min. The T_g was calculated as the inflection point of the heat flow versus temperature curve. The T_g values for all blends were fitted using the Gordon-Taylor equation (Eq. (1)).

The T_g of moisturized pullulan/trehalose blends, *i.e.* after 72 h exposure to different RH's using saturated salt solutions (Table 1) at 25 °C, was determined using a Q2000 DSC. An amount of 2 to 5 mg was accurately weighed in Tzero aluminum pans (TA Instruments, Ghent, Belgium) and directly hermetically closed using a Tzero lid (TA Instruments, Ghent, Belgium). After equilibration at -20 °C for non-collapsed samples and -50 °C for collapsed samples, the temperature was raised to 150 °C at a rate of 20 °C/min. Next, the samples were cooled to the initial temperature at a rate of 20 °C/min and the first heating step was repeated. Reported T_g s were calculated from the thermograms of the second heating step. The inflection point in the heat flow versus temperature curve was taken as the T_g .

2.5. Storage stability testing

The storage stability of β -galactosidase in pullulan/trehalose blends and without sugar was assessed under different conditions. The storage stability of closed vials was tested at 60 °C and 30 °C. Open vials were used for storage stability testing at 30 °C and 56% RH, using a saturated NaBr solution. The enzymatic activity of β -galactosidase was measured immediately after freeze-drying and after 1, 2 and 4 weeks of storage (3

independent experiments per time point). The remaining enzymatic activity immediately after freeze-drying of β -galactosidase without sugar, which was 83%, was used to relate all other activities to (normalization to 100%).

2.6. Enzymatic activity of β -galactosidase

The enzymatic activity of β -galactosidase was determined using a kinetic enzymatic assay, based on the conversion of the substrate *ortho*-nitrophenyl- β -galactoside (colorless) to *ortho*-nitrophenol (yellow) by β -galactosidase. In brief, samples of 5 μ g/mL were prepared in 50 mM phosphate buffer supplemented with 0.1% BSA and 1 mM $MgCl_2$. Next, 20 μ L sample was pipetted into each well of a 96-well plate (Greiner, F-shape), followed by 200 μ L $MgCl_2$ solution (1.4 mM $MgCl_2$ in 0.1 M phosphate buffer, pH 7.3). The plate was incubated at 37 °C for 10 min after which 20 μ L of 50 mM *ortho*-nitrophenyl- β -galactoside was added to the wells. The conversion to *ortho*-nitrophenol was determined by measuring the absorbance at 405 nm every 30 s for 15 min at 37 °C (Synergy HT Microplate Reader, BioTek Instruments, Winooski, VT). The activity of β -galactosidase in units/mg was calculated from the slope of this conversion.

2.7. Data analysis

All the data are represented as mean of $n = 3 \pm$ SD when applicable. The graphs and curve fittings were performed using Graphpad Prism version 6.0 (GraphPad Prism Software, Inc., La Jolla, CA, USA).

3. Results

3.1. Water sorption

The vapor sorption isotherms of the different pullulan/trehalose blends were comparable between 0% and 40% RH. At higher RH values, differences became apparent. Trehalose showed a maximum weight gain at 50% RH of 12.4% (Fig. 2). At higher RH, the weight gain slightly decreased to around 11.3% . This pattern is accordance with the formation of the crystalline trehalose dehydrate (Costantino, Curley, Wu, & Hsu, 1998; Hinrichs et al., 2001). The addition of 17% and 33% pullulan delayed the crystallization of trehalose to 60% RH (Fig. 2). Ultimately, at equal amounts of pullulan and trehalose, crystallization of trehalose was fully prevented within the time frame of the experiment. At higher pullulan contents, similar behavior was observed and trehalose did not crystallize (Fig. S1).

3.2. Glass transition Temperature (T_g)

The thermograms of pullulan/trehalose solutions at all ratios showed a single T_g' (data not shown), which demonstrates the formation of a homogeneous blend of the two sugars in the maximally freeze concentrated fraction. The T_g' values of all blends were well above the primary drying temperature of the freeze-drying process (Fig. 3a). No

Table 1
Relative humidity values over saturated salt solutions at 25 °C (Greenspan, 1977; Winston et al., 1960) used in this study.

Salt	Relative Humidity (%)
Lithium Chloride	11.3
Potassium Acetate	22.5
Magnesium Chloride	32.8
Potassium Carbonate	43.2
Sodium Bromide	57.6
Sodium Nitrite	64.0
Ammonium Chloride	78.6
Barium Chloride	90.0

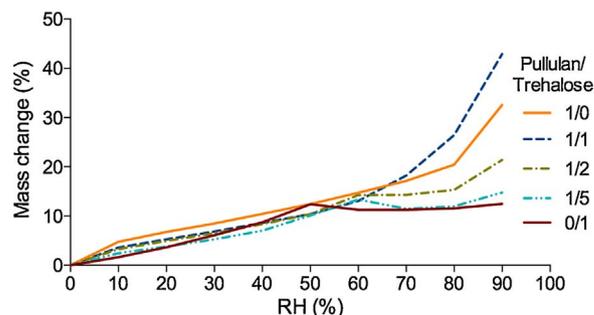


Fig. 2. Vapor sorption isotherms of freeze-dried pullulan/trehalose blends at 25 °C.

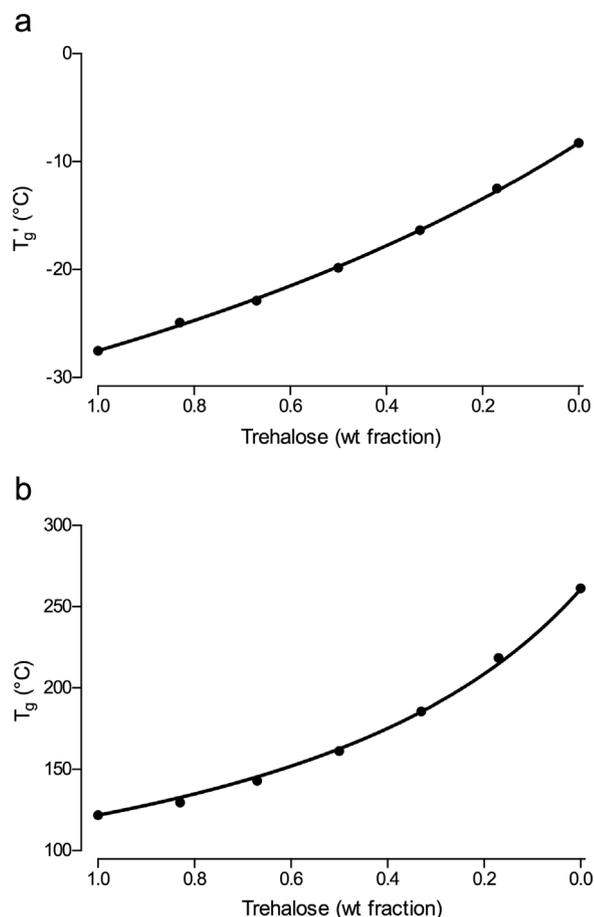


Fig. 3. Glass transition temperatures of maximally freeze concentrated pullulan/trehalose solutions T_g' (a) and freeze-dried pullulan/trehalose blends T_g (b), fitted using the Gordon-Taylor equation.

collapse was observed in any of the formulations after freeze-drying. Fitting of the T_g' values with the Gordon-Taylor equation yielded a k value of 0.6818 ($R^2 = 0.9994$).

Also, the thermograms of freeze-dried pullulan/trehalose solutions at all ratios showed a single T_g (data not shown), indicating that the sugars remained homogeneously mixed during drying. The T_g values of freeze-dried pullulan/trehalose blends are depicted in Fig. 2b. Pullulan showed a T_g of 261 °C (Fig. 3b), which is substantially higher than (predicted) values found elsewhere (Biliaderis, Lazaridou, & Arvanitoyannis, 1999; Biliaderis, Lazaridou, Mavropoulos, & Barbayiannis, 2002; Diab, Biliaderis, Gerasopoulos, & Sfakiotakis, 2001; Vuddanda et al., 2017). This difference could be due to residual water or lower molecular weight of the pullulan investigated. Trehalose showed a plasticizing effect on pullulan and caused the T_g to drop with increasing weight fraction. The T_g of freeze-dried trehalose was 121 °C (Fig. 3b), which is similar to values found in literature (Tonnis et al., 2015; Vandenheuvel et al., 2014). These values were fitted using the Gordon-Taylor equation and resulted in a k value of 0.4163 ($R^2 = 0.9980$).

Exposure of freeze-dried pullulan/trehalose blends to different RH conditions (Table 1) during 72 h resulted in a decrease of the T_g in all formulations (Fig. 4), which obviously can be ascribed to the plasticizing effect of adsorbed water. Collapse of some formulations was visually observed, with a trend towards increasing RHs and increasing trehalose content. Formulations containing only pullulan did not collapse during exposure to any of the conditions. Some formulations that were collapsed after exposure still showed a T_g (marked with * in Fig. 4). However, for the remainder of the collapsed formulations partial or complete crystallization of trehalose occurred and no T_g s were detectable (Table 2). The occurrence of crystallization in the

formulations at pullulan/trehalose weight ratio of 0/1, 1/5 and 1/2 is in agreement with the DVS measurements.

3.3. Storage stability

The stability of β -galactosidase, freeze-dried without sugar and in different blends of pullulan/trehalose, was tested under different storage conditions. Without sugar, no activity loss was observed after freeze-drying, however, a rapid decline in activity of β -galactosidase was observed at 60 °C (Fig. 5a), with only 0.5% residual activity after 1 week. At 30 °C, the stability of β -galactosidase showed similar behavior at both 0% and 56% RH conditions. As can be expected, the decrease in activity was slower at 30 °C than at 60 °C. Nonetheless, after two weeks the remaining enzymatic activity of β -galactosidase was only 3% for the samples exposed to 56% RH and 14% for samples exposed to 0% RH (Fig. 5a). After four weeks exposure to any of the tested conditions, the remaining activity of β -galactosidase was negligible. All freeze-dried formulations containing sugars performed better after four weeks of storage than freeze-dried β -galactosidase without sugar (Fig. 5), indicating the stabilizing capabilities of both sugars regardless of their ratios.

The process stability of freeze-drying β -galactosidase in pullulan/trehalose blends was between 83% for pullulan only and gradually increased with decreasing pullulan/trehalose ratios until 96% for trehalose only (Fig. 5b). During storage at any of the conditions, the enzymatic activity of β -galactosidase decreased in all formulations (Fig. 5bcd). At 0% RH conditions, enzymatic activity loss was more severe at higher temperature and higher pullulan content (Fig. 5bc). Hence, the trehalose only formulation performed best in 0% RH conditions. In contrast, when exposed to 56% RH at 30 °C, the formulations with a pullulan/trehalose weight ratio of 2/1, 1/1, 1/2 and 1/5 all performed better than the trehalose only formulation (Fig. 5d). In particular, the blends with pullulan/trehalose weight ratio 1/2 and 1/5 showed the same remaining activity of 76% and 83%, respectively, compared to 0% RH, even though these formulations were collapsed during storage. DSC measurements of pullulan/trehalose blends without HEPES buffer and β -galactosidase exposed to 30 °C/56% RH only showed crystallization of trehalose in the trehalose only formulation (Table S1). The T_g s of the formulations with a pullulan/trehalose weight ratio of 1/2 and 1/5 were below storage temperature, *i.e.* 25.7 and 15.5 °C, respectively. It was assumed that the very small quantities of HEPES buffer and β -galactosidase in the storage stability samples (1.3 wt-% in total) had no effect on T_g .

4. Discussion

In this study, we investigated the applicability of pullulan as (an additive to) sugar glass matrices for stabilization of proteins by assessing various physicochemical properties of pullulan and pullulan/trehalose blends. In addition, we evaluated the storage stability of β -galactosidase, encapsulated in pullulan/trehalose blends, under different temperature and humidity conditions. Our results show that pullulan has some advantageous properties for protein stabilization, most importantly its very high T_g which amply remains above room temperature even when exposed to high RH. This was demonstrated by the improved storage stability of the model protein β -galactosidase after encapsulation in a pullulan sugar glass. The stability of the protein could even be further improved by using a blend of trehalose with pullulan as sugar glass matrix.

Since pullulan is a bulky molecule, it may not be able to provide for an optimal molecular packing around proteins, thus preventing optimal local vitrification and water replacement (Mensink et al., 2017), despite the benefits of its high T_g . Basically, the application of a blend consisting of a small disaccharide and a large polysaccharide combines the advantages of both a tight molecular packing (gaps left by the polysaccharide are filled by the disaccharide) (Tonnis et al., 2015) and a

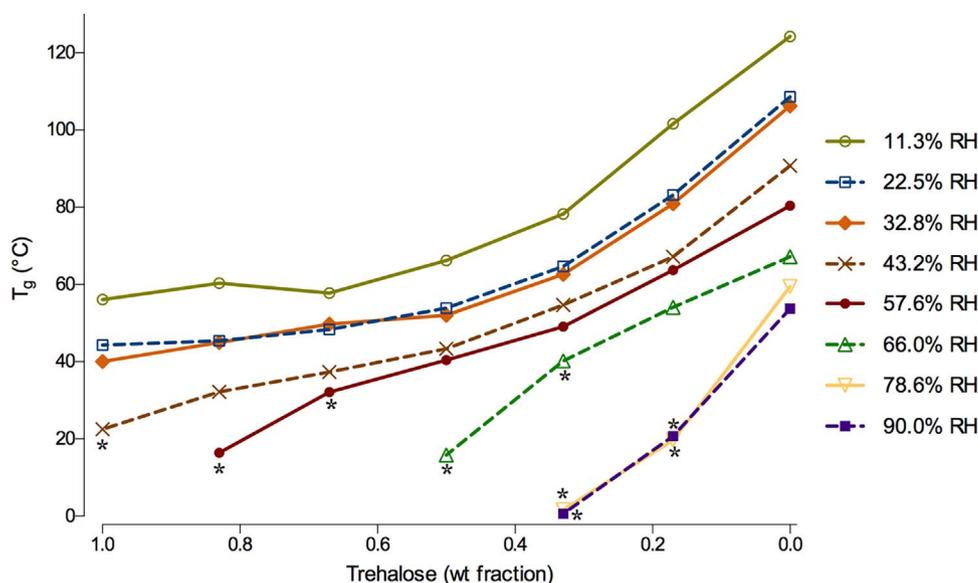


Fig. 4. Glass transition temperatures (T_g) of pullulan/trehalose blends after 72 h exposure to different relative humidity conditions at 25 °C. Data points marked with (*) indicate collapsed samples.

Table 2

Crystallinity of trehalose in pullulan/trehalose blends after 72 h exposure to different relative humidity conditions, expressed as a percentage. To calculate the percentage of crystallized trehalose, the melting enthalpy of trehalose as determined by DSC was compared to the melting enthalpy of fully crystalline trehalose. Values are corrected for trehalose content in the formulations.

	Pullulan/trehalose			
	1/1	1/2	1/5	0/1
43.2% RH	0.0	0.0	0.0	2.8
57.6% RH	0.0	0.0	0.0	96.6
64.0% RH	0.0	6.7	78.5	99.8
78.6% RH	53.0	61.6	84.1	105.3
90.0% RH	56.1	69.2	83.3	96.5

high T_g (from the polysaccharide). These combinations of a polysaccharide and disaccharide have been described before (Allison et al., 2000; Tonnis et al., 2015). Since disaccharides have a plasticizing effect on polysaccharides, a high T_g of the polysaccharide is preferable to maintain a high T_g after blending, especially because the T_g will additionally be affected by residual and absorbed water. Therefore, we intended to “titrate” pullulan with trehalose to investigate the effects on the physicochemical properties and to find an optimum blend composition for protein stabilization. We selected pullulan (200–300 kDa average molecular weight) because we found that this polysaccharide has an exceptionally high T_g of 261 °C compared to other polysaccharides: the T_g s of dextran 70 kDa and maltodextrin 19 kDa are about 224 °C and 200 °C, respectively (Kilburn, Claude, Schweizer, Alam, & Ubbink, 2005; Tonnis et al., 2015).

As expected, adsorbed water had a major plasticizing effect on the pullulan/trehalose blends, which is reflected in decreased T_g values (Fig. 4). It is noteworthy that the T_g of pullulan, even after exposure to 90% RH, remains well above room temperature. Also, it has become apparent that collapse of samples consisting of the pullulan/trehalose blend do not necessarily indicate trehalose crystallization, although the T_g of these formulations dropped below room temperature and would thereby probably impair protein stabilization. However, the possibility that crystallization will occur upon longer exposure to high RH conditions cannot be excluded.

DVS was found to be an elegant method to predict crystallization of trehalose in these binary glasses, as the DSC results and visual inspection of the samples exposed to different RHs generally correlated well with the DVS results. However, DSC of the blend with a pullulan/

trehalose weight ratio of 1/1 indicated that trehalose crystallized when exposed to 78.6% RH and higher while with DVS this effect was not observed. Possibly, the high amount of water absorbed by pullulan during DVS at high RHs concealed the crystallization pattern as seen with other formulations using this method.

The process stability of the freeze-dried formulations clearly decreased with increased pullulan content. However, as mentioned above, the steric hindrance caused by the large pullulan molecules may impair a tight packing of the sugar around the protein. Consequently, the protein might be less protected from stresses during freeze-drying. Indeed, it has been shown that the addition of a disaccharide to large polysaccharides results in an increase in process stability (Santagapita et al., 2015) and a better preservation of protein secondary structure after freeze-drying (Allison et al., 2000; Garzon-Rodriguez et al., 2004).

The storage stability results clearly show that trehalose is an optimal stabilizer under condition free from any moisture (0% RH, closed vials) at 30 °C and 60 °C, despite having the lowest T_g . This is in line with earlier findings for dextran 70 kDa and trehalose blends (Tonnis et al., 2015). In anhydrous conditions, the degree of vitrification of the protein in the sugar glass determines its stability. The vitrification is assumed to be best when the protein is tightly packed with sugar molecules, which would be the case for the disaccharide containing formulations.

However, when moisture is present such as in the 56% RH/30 °C condition we tested, trehalose did not perform best. The same was also observed for trehalose/dextran 70 kDa systems (Santagapita et al., 2015). Trehalose crystallization has been known to occur at RH values of 45% at 25 °C (Diab et al., 2001) and higher temperatures (Jórátlaczovich, Katona, Aigner, & Szabó-révész, 2016), and also at a low temperature of 4 °C and 54% RH, trehalose crystallizes (Vandenheuvel et al., 2014). From these previous studies, combined with the DVS and DSC measurements of the present study, it can be concluded that the trehalose only formulation crystallized at 30 °C/56% RH. It is well known that a sugar loses its protective properties once it is crystallized (Eriksson et al., 2002), and it is the most likely cause for the suboptimal stabilization of trehalose when moisture is present. Here, we assume that the presence of the protein and buffer did not disturb crystallization, which is highly plausible as these components are only present in very small quantities (together 1.3 wt-% of the formulation).

At 56% RH/30 °C storage conditions, the formulations with a pullulan/trehalose weight ratio of 2/1, 1/1, 1/2 and 1/5 outperformed the trehalose only formulation. This is in agreement with the DVS measurements of the blends with a pullulan/trehalose weight ratio of 2/1

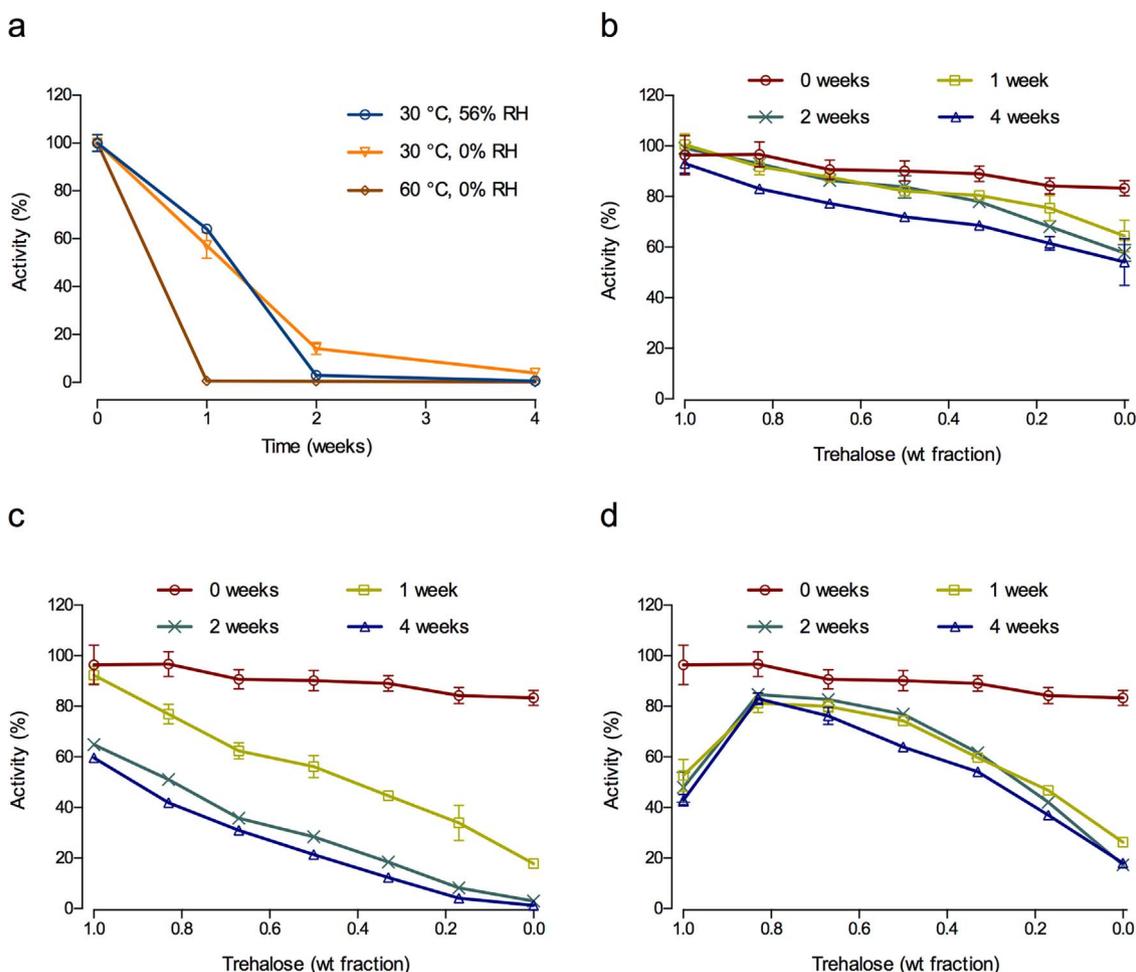


Fig. 5. Stability of freeze-dried β -galactosidase, with and without various pullulan/trehalose mixtures, at different storage conditions up to 4 weeks. Stability of freeze-dried β -galactosidase formulated without sugars during storage up to 4 weeks at 30 °C/56% RH, 30 °C/0% RH and 60 °C/0% RH (a). Process stability (no exposure) of β -galactosidase, freeze-dried in different pullulan/trehalose blends and storage stability up to 4 weeks of these formulations at different conditions: 30 °C/0% RH (b), 60 °C/0% RH (c), and 30 °C/56% RH (d).

and 1/1, which showed that the presence of pullulan prevented crystallization of trehalose, although these measurements were done at a slightly lower temperature, *i.e.* 25 °C, than the storage stability study, *i.e.* 30 °C. However, DSC measurements clearly showed that the T_g s of the 2/1 and 1/1 pullulan/trehalose formulations were above storage temperature (Table S1). Hence, these formulations remained in the glassy state and therefore provided better protection than the trehalose only formulation.

In contrast, blends with a pullulan/trehalose weight ratio of 1/2 and 1/5 had a T_g below storage temperature. These formulations were found to collapse during exposure to 56% RH and 30 °C. Nevertheless, under these humid conditions, the 1/2 and 1/5 blends remarkably provided the best storage stability of β -galactosidase. The most likely explanation would be that crystallization of trehalose was prevented or delayed due to the presence of pullulan, which is supported by the DVS measurements at 25 °C. In addition, the collapse and the absorbed water may have caused a rearrangement of the sugar molecules other than crystallization. Such molecular rearrangement potentially results in tighter packing of sugar molecules around the protein than before collapse, thereby providing excellent stability of the protein by improved water replacement. This may imply that in this particular case vitrification of the protein is of secondary importance. Furthermore, the stabilizing capacity of these formulations is in agreement with the work of Schersch and coworkers, who showed that the long term stability of several proteins was only slightly affected in intentionally collapsed amorphous cakes using collapse-drying (Schersch et al., 2010, 2013). Obviously, the protective properties of pullulan itself may also have

contributed to the stability.

5. Conclusion

The assessment of the biocompatible polysaccharide pullulan (M_w 200–300 kDa) as a sugar glass for protein stabilization yielded a very high T_g of 261 °C, which is an excellent attribute for protein stabilization. Yet, pullulan's bulky nature prevents a tight molecular packing around proteins, which resulted in a process and storage stability of the model protein β -galactosidase that was inferior to the disaccharide trehalose at conditions free from moisture (0% RH). However, when moisture is present (*e.g.* 56% RH), as may occur in daily practice, blends of pullulan and trehalose outperformed trehalose alone. Under these conditions, the presence of pullulan increased the T_g of the formulations compared to trehalose alone and furthermore, pullulan prevented crystallization of trehalose. Hence, pullulan/trehalose blends combine the strengths of both sugars, namely the high T_g of pullulan and the tight molecular packing of trehalose. Altogether, our results show the potential of pullulan/trehalose blends for application as a pharmaceutical excipient in stabilizing proteins in solid form.

Acknowledgements

The authors thank Michelle Wijma for her preliminary work on the characterization of pullulan. This work was supported by The Northern Netherlands Province Alliance (grant number T2002) and the China Scholarship Council.

Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at <http://dx.doi.org/10.1016/j.carbpol.2017.08.084>.

References

- Allison, S. D., Chang, B., Randolph, T. W., & Carpenter, J. F. (1999). Hydrogen bonding between sugar and protein is responsible for inhibition of dehydration-induced protein unfolding. *Archives of Biochemistry and Biophysics*, *365*(2), 289–298.
- Allison, S. D., Manning, M. C., Randolph, T. W., Middleton, K., Davis, A., & Carpenter, J. F. (2000). Optimization of storage stability of lyophilized actin using combinations of disaccharides and dextran. *Journal of Pharmaceutical Sciences*, *89*(2), 199–214.
- Balcão, V. M., & Vila, M. M. D. C. (2014). Structural and functional stabilization of protein entities: state-of-the-art. *Advanced Drug Delivery Reviews*, *93*, 25–41.
- Biliaderis, C. G., Lazaridou, A., & Arvanitoyannis, I. (1999). Glass transition and physical properties of polyol-plasticized pullulan-starch blends at low moisture. *Carbohydrate Polymers*, *40*(1), 29–47.
- Biliaderis, C. G., Lazaridou, A., Mavropoulos, A., & Barbayiannis, N. (2002). Water plasticization effects on crystallization behavior of lactose in a co-lyophilized amorphous polysaccharide matrix and its relevance to the glass transition. *International Journal of Food Properties*, *5*(2), 463–482.
- Chang, L., Shepherd, D., Sun, J., Ouellette, D., Grant, K. L., Tang, X., & Pikal, M. J. (2005). Mechanism of protein stabilization by sugars during freeze-drying and storage: Native structure preservation, specific interaction, and/or immobilization in a glassy matrix? *Journal of Pharmaceutical Sciences*, *94*(7), 1427–1444.
- Cheng, K. C., Demirci, A., & Catchmark, J. M. (2011). Pullulan: Biosynthesis, production, and applications. *Applied Microbiology and Biotechnology*, *92*(1), 29–44.
- Cicerone, M. T., Pikal, M. J., & Qian, K. K. (2015). Stabilization of proteins in solid form. *Advanced Drug Delivery Reviews*, *93*, 14–24.
- Costantino, H. R., Curley, J. G., Wu, S., & Hsu, C. C. (1998). Water sorption behavior of lyophilized protein-sugar systems and implications for solid-state interactions. *International Journal of Pharmaceutics*, *166*(2), 211–221.
- Crowe, J. H., Carpenter, J. F., & Crowe, L. M. (1998). The Role of Vitrification in Anhydrobiosis. *Annu. Rev. Physiol.* *60*, 73–103.
- Diab, T., Biliaderis, C. G., Gerasopoulos, D., & Sfakiotakis, E. (2001). Physicochemical properties and application of pullulan edible films and coatings in fruit preservation. *Journal of the Science of Food and Agriculture*, *81*(10), 988–1000.
- Dionisio, M., Cordeiro, C., Remunan-Lopez, C., Seijo, B., Da Costa, A. M. R., & Grenha, A. (2013). Pullulan-based nanoparticles as carriers for transmucosal protein delivery. *European Journal of Pharmaceutical Sciences*, *50*(1), 102–113.
- Eriksson, H. J. C., Hinrichs, W. L. J., Van Veen, B., Somsen, G. W., De Jong, G. J., & Frijlink, H. W. (2002). Investigations into the stabilisation of drugs by sugar glasses: I. Tablets prepared from stabilised alkaline phosphatase. *International Journal of Pharmaceutics*, *249*(1–2), 59–70.
- Garsuch, V. I. (2009). *Preparation and characterization of fast-dissolving oral films for pediatric use*. Düsseldorf: Heinrich-Heine University.
- Garzon-Rodriguez, W., Koval, R. L., Chongprasert, S., Krishnan, S., Randolph, T. W., Warne, N. W., & Carpenter, J. F. (2004). Optimizing Storage Stability of Lyophilized Recombinant Human Interleukin-11 with Disaccharide/Hydroxyethyl Starch Mixtures. *Journal of Pharmaceutical Sciences*, *93*(3), 684–696.
- Greenspan, L. (1977). Humidity Fixed Points of Binary Saturated Aqueous Solutions. *Journal of Research of the National Bureau of Standards – A. Physics and Chemistry*, *81A*(1).
- Hinrichs, W. L., Prinsen, M. G., & Frijlink, H. W. (2001). Inulin glasses for the stabilization of therapeutic proteins. *International Journal of Pharmaceutics*, *215*(1–2), 163–174.
- Jahanshahi-Anbuhi, S., Kannan, B., Leung, V., Pennings, K., Liu, M., Carrasquilla, C., ... Filipe, C. D. M. (2016). Simple and ultrastable all-inclusive pullulan tablets for challenging bioassays. *Chem. Sci.* *7*(3), 2342–2346.
- Jiskoot, W., Randolph, T. W., Volkin, D. B., Middaugh, C. R., Schöneich, C., Winter, G., ... Carpenter, J. F. (2012). Protein instability and immunogenicity: Roadblocks to clinical application of injectable protein delivery systems for sustained release. *Journal of Pharmaceutical Sciences*, *101*(3), 946–954.
- Jórárt-laczkovich, O., Katona, G., Aigner, Z., & Szabó-révész, P. (2016). Investigation of recrystallization of amorphous trehalose through hot-humidity stage X-ray powder diffraction. *European Journal of Pharmaceutical Sciences*, *95*, 145–151.
- Kilburn, D., Claude, J., Schweizer, T., Alam, A., & Ubbink, J. (2005). Carbohydrate polymers in amorphous states: An integrated thermodynamic and nanostructural investigation. *Biomacromolecules*, *6*(2), 864–879.
- Lai, M. C., & Topp, E. M. (1999). Solid-state chemical stability of proteins and peptides. *Journal of Pharmaceutical Sciences*, *88*(5), 489–500.
- Manning, M. C., Chou, D. K., Murphy, B. M., Payne, R. W., & Katayama, D. S. (2010). Stability of protein pharmaceuticals: an update. *Pharmaceutical Research*, *27*(4), 544–575.
- Mensink, M. A., Frijlink, H. W., van der Voort Maarschalk, K., & Hinrichs, W. L. J. (2017). How sugars protect proteins in the solid state and during drying (review): Mechanisms of stabilization in relation to stress conditions. *European Journal of Pharmaceutics and Biopharmaceutics*, *114*, 288–295.
- Mensink, M. A., Van Bockstal, P.-J., Pieters, S., De Meyer, L., Frijlink, H. W., van der Voort Maarschalk, K., ... De Beer, T. (2015). In-line near infrared spectroscopy during freeze-drying as a tool to measure efficiency of hydrogen bond formation between protein and sugar, predictive of protein storage stability. *International Journal of Pharmaceutics*, *496*(2), 792–800.
- Mitragotri, S., Burke, P. A., & Langer, R. (2014). Overcoming the challenges in administering biopharmaceuticals: formulation and delivery strategies. *Nature Reviews Drug Discovery*, *13*(9), 655–672.
- Prajapati, V. D., Jani, G. K., & Khanda, S. M. (2013). Pullulan: An exopolysaccharide and its various applications. *Carbohydrate Polymers*, *95*(1), 540–549.
- Ratanji, K. D., Derrick, J. P., Dearman, R. J., & Kimber, I. (2014). Immunogenicity of therapeutic proteins: Influence of aggregation. *Journal of Immunotoxicology*, *11*(2), 99–109.
- Santagapita, P. R., Mazzobre, M. F., Buera, M. P., Ramirez, H. L., Brizuela, L. G., Corti, H. R., & Villalonga, R. (2015). Impact of supramolecular interactions of dextran- β -cyclodextrin polymers on invertase activity in freeze-dried systems. *Biotechnology Progress*, *31*(3), 791–798.
- Saxenian, H. (2017). *Immunization financing: a resource guide for advocates, policy-makers, and program managers*. Resource Guide by Results for Development Institute: Washington, DC.
- Schersch, K., Betz, O., Garidel, P., Muehla, S., Bassarab, S., & Winter, G. (2010). Systematic Investigation of the Effect of Lyophilizate Collapse on Pharmaceutically Relevant Proteins I: Stability after Freeze-Drying. *Journal of Pharmaceutical Sciences*, *99*, 2256–2278.
- Schersch, K., Betz, O., Garidel, P., Muehla, S., Bassarab, S., & Winter, G. (2013). Systematic investigation of the effect of lyophilizate collapse on pharmaceutically relevant proteins III: Collapse during storage at elevated temperatures. *European Journal of Pharmaceutics and Biopharmaceutics*, *85*(2), 240–252.
- Shingel, K. I. (2004). Current knowledge on biosynthesis, biological activity, and chemical modification of the exopolysaccharide, pullulan. *Carbohydrate Research*, *339*(3), 447–460.
- Tonnis, W. F., Mensink, M. A., de Jager, A., van der Voort Maarschalk, K., Frijlink, H. W., & Hinrichs, W. L. J. (2015). Size and Molecular Flexibility of Sugars Determine the Storage Stability of Freeze-Dried Proteins. *Molecular Pharmaceutics*, *12*(3), 684–694.
- Vandenhoevel, D., Meeus, J., Lavigne, R., & Van Den Mooter, G. (2014). Instability of bacteriophages in spray-dried trehalose powders is caused by crystallization of the matrix. *International Journal of Pharmaceutics*, *472*(1–2), 202–205.
- Vuddanda, P. R., Montenegro-Nicolini, M., Morales, J. O., & Velaga, S. (2017). Effect of plasticizers on the physico-mechanical properties of pullulan based pharmaceutical oral films. *European Journal of Pharmaceutical Sciences*, *96*, 290–298.
- Winston, P. W., Bates, D. H., Ecology, S., Jan, N., Bovbjerg, R. V., & Glynn, P. W. (1960). Saturated Solutions For the Control of Humidity in Biological Research. *Ecology*, *41*(1), 232–237.
- Xi, K., Tabata, Y., Uno, K., Yoshimoto, M., Kishida, T., Sokawa, Y., & Ikada, Y. (1996). Liver targeting of interferon through pullulan conjugation. *Pharmaceutical Research*.