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## Solid state stabilization of proteins by sugars

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Size and molecular  
flexibility of sugars  
determine the storage  
stability of freeze-dried  
proteins

2

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**ABSTRACT**

Protein-based biopharmaceuticals are generally produced as aqueous solutions and stored refrigerated to obtain sufficient shelf life. Alternatively, proteins may be freeze-dried in the presence of sugars to allow storage stability at ambient conditions for prolonged periods. However, to act as a stabilizer, these sugars should remain in the glassy state during storage. This requires a sufficiently high glass transition temperature (T<sub>g</sub>). Furthermore, the sugars should be able to replace the hydrogen bonds between the protein and water during drying. Frequently used disaccharides are characterized by a relatively low T<sub>g</sub>, rendering them sensitive to plasticizing effects of residual water, which strongly reduces the T<sub>g</sub> values of the formulation. Larger sugars generally have higher T<sub>g</sub>s, but it is assumed that these sugars are limited in their ability to interact with the protein due to steric hindrance.

In this chapter, the size and molecular flexibility of sugars was related to their ability to stabilize proteins. Four diverse proteins varying in size from 6 kDa to 540 kDa were freeze-dried in the presence of different sugars varying in size and molecular flexibility. Subsequently, the different samples were subjected to an accelerated stability test. Using protein specific assays and intrinsic fluorescence, stability of the proteins was monitored. It was found that the smallest sugar (disaccharide trehalose) best preserved the proteins, but also that the T<sub>g</sub> of the formulations was only just high enough to maintain sufficient vitrification. When trehalose-based formulations are exposed to high relative humidities, water uptake by the product reduces the T<sub>g</sub>s too much. In that respect, sugars with higher T<sub>g</sub>s are desired. Addition of polysaccharide dextran 70 kDa to trehalose greatly increased the T<sub>g</sub> of the formulation. Moreover, this combination also improved the stability of the proteins compared to dextran only formulations. The molecularly flexible oligosaccharide inulin 4 kDa provided better stabilization than the similar sized but molecularly rigid oligosaccharide dextran 6 kDa.

In conclusion, the results of this study indicate that size and molecular flexibility of sugars affect their ability to stabilize proteins. As long as they maintain vitrified, smaller and molecularly more flexible sugars are less affected by steric hindrance and thus better capable at stabilizing proteins.

## INTRODUCTION

Protein-based biopharmaceuticals such as recombinant monoclonal antibodies, subunit vaccines, cytokines, and hormones are generally produced as aqueous solutions. Storage of these often expensive solutions under ambient conditions may lead to fast degradation of the protein, which results in the formation of products that are inactive or even elicit unwanted immune responses. To obtain sufficient shelf life, these products are usually stored and transported refrigerated. The dependence on this so-called “cold-chain” makes these products even more expensive and transport to rural areas in tropical developing countries is often impossible. One of the potentially most effective strategies to improve the stability of proteins is to bring them in the dry state. Most degradation pathways require molecular mobility of the protein. In the dry state this molecular mobility is strongly reduced, resulting in increased stability. Freeze-drying is one of the techniques used to dry protein solutions.<sup>1</sup> However, during freeze-drying, proteins are subjected to freezing and drying stresses. It is well known that sugars can be used as stabilizing excipients to prevent degradation resulting from these stresses.<sup>1</sup>

A concomitant advantage of using sugars is that they can also contribute to an improved storage stability of the dried proteins. Two main mechanisms have been described to explain the stabilization of proteins by sugars: water replacement<sup>2</sup>, and vitrification.<sup>3,4</sup> Proteins (partially) lose their hydration shells during freezing and drying, which can lead to the formation of intramolecular hydrogen bonds within the proteins, changing their three-dimensional structures. When sugars are added prior to drying, the hydrogen bonds between the protein and water (the hydration shell) are gradually replaced by hydrogen bonds between the protein and hydroxyl groups of the sugar during freeze-drying, thereby conserving the protein's three-dimensional structure.<sup>5-7</sup> According to the vitrification theory, the molecular mobility of the protein is strongly reduced when it is incorporated in a sugar matrix in the glassy state, resulting in a reduced degradation rate of the protein. Both water replacement and vitrification require close contact between the sugar and the protein at a molecular level to stabilize the protein.

It is well established that the water replacement and vitrification theories alone can not explain the stabilization achieved by sugars.<sup>4,8</sup> More recent work has refined these theories and related local vitrification in the form of fast  $\beta$ -relaxation in the solid state to protein stability.<sup>8</sup> It was found that the disaccharide trehalose stabilized model proteins by reduction of their molecular motion both during freeze-drying and in the freeze-dried solid.<sup>9,10</sup> Sugar molecules are significantly larger than water molecules and are therefore not able to fully replace water-protein hydrogen bonds.<sup>10</sup> This is in line with the water entrapment hypothesis, a refinement of the water replacement hypothesis that states that some residual water remains present around the protein, rather than that the sugars

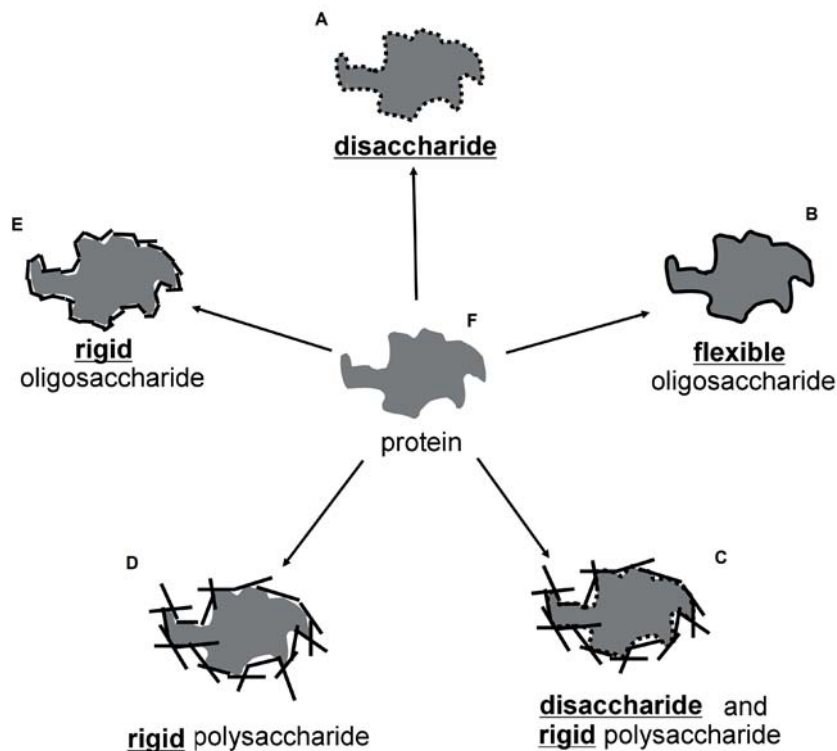
directly interact with the protein.<sup>11</sup> It is now possible to relate these refined hypotheses to each other using the slaving model, which states that mobility of the protein is governed by the bulk matrix and its hydration.<sup>12</sup> The reader should be aware of these refinements, but for clarity purposes the long established terms vitrification and water replacement will be used throughout this chapter.

Various types of sugars can be used to stabilize proteins during freeze-drying and subsequent storage. To act as an appropriate stabilizer, the sugar should meet at least two important requirements. Firstly, the sugar should remain in the glassy state during storage, favoring sugars with a high glass transition temperature (T<sub>g</sub>). At temperatures above the T<sub>g</sub>, the sugar is in the rubbery state, displaying high molecular mobility by which vitrification is compromised. In addition, crystallization of the sugar in the rubbery state occurs easily. Crystallization can damage the protein through mechanical stresses but also results in a loss of the close contact between the sugar and proteins and therefore a loss of stability. Secondly, the sugar should contain no or only a very limited number of reducing groups. Reducing groups can react with amine groups of proteins to form a N-substituted glycosylamine, which is the first step of a cascade of reactions also referred to as the Maillard reaction or browning.<sup>13</sup> This reaction can proceed fast in the liquid state, but was also reported to occur in the solid state.<sup>14</sup>

Sugars can be categorized into mono-, di-, oligo- and polysaccharides. Monosaccharides like glucose, fructose, and galactose are not suitable as protein stabilizers during storage since these sugars have a low T<sub>g</sub> (<40 °C) and contain reducing groups. The disaccharides sucrose and trehalose possess a much higher T<sub>g</sub> (77 °C for sucrose and 121 °C for trehalose<sup>15</sup>) and do not contain reducing groups and are therefore often used as protein stabilizers.<sup>16</sup> Water, be it residual moisture after freeze-drying or water taken up during storage, acts as a plasticizer, which lowers the T<sub>g</sub> strongly. This could lower the T<sub>g</sub> to below the storage temperature, resulting in a poor stabilization or even destabilization as described above. An advantage of oligosaccharides like inulin and dextran is their high T<sub>g</sub> values (the T<sub>g</sub> values of moisture free inulin with an average molecular weight of 4 kDa and dextran 5 kDa are 157 °C and 176 °C, respectively)<sup>15</sup>. These temperatures lie far above any usual storage condition. Even if some residual moisture is present after freeze-drying, the storage temperature will still be significantly lower than the T<sub>g</sub>. Polysaccharides exhibit even higher T<sub>g</sub>s. As described by the Fox-Flory equation, the T<sub>g</sub> increases with the molecular weight of the polymer.<sup>17</sup>

A downside of many high molecular weight sugars is the combination of a large size with the limited flexibility of the molecular chains. Due to this combination, hydrogen bond interactions with proteins are sterically hindered and efficient vitrification at the surface of the protein will become difficult to achieve.<sup>6</sup> This is schematically shown in figure 2.1, which shows the interaction of the adsorbing part of the sugar with the protein. It should be noted

that this illustration oversimplifies the stabilization of sugars on proteins, in order to illustrate the steric effects to the reader and that as explained in the introduction. Rigid polysaccharides are able to interact with the protein surface, but leave open gaps (figure 2.1D). For sugars with high molecular weights and a limited flexibility these gaps are large (figure 2.1D) compared to the smaller sugars (figure 2.1A). Addition of a polysaccharide with a high Tg to the disaccharide might provide benefit by combining a proper coating with a high Tg (figure 2.1C).<sup>18</sup> Such combinations have been previously described; i.e. disaccharides in combination with hydroxyethyl starch or dextran, but the mechanisms of stabilization were not investigated.<sup>18,19</sup> The obvious downside of such an approach is that the small disaccharide acts as a plasticizer for the larger polymer. For that reason the Tg of the polysaccharide has to be high enough to compensate for this Tg reduction. Since oligosaccharides are smaller than polysaccharides, oligosaccharides might achieve a more compact coating (compare figure 2.1D and E) and with that a more stable formulation. We hypothesize that compactness of the coating of proteins by oligosaccharides is also dependent on the molecular flexibility

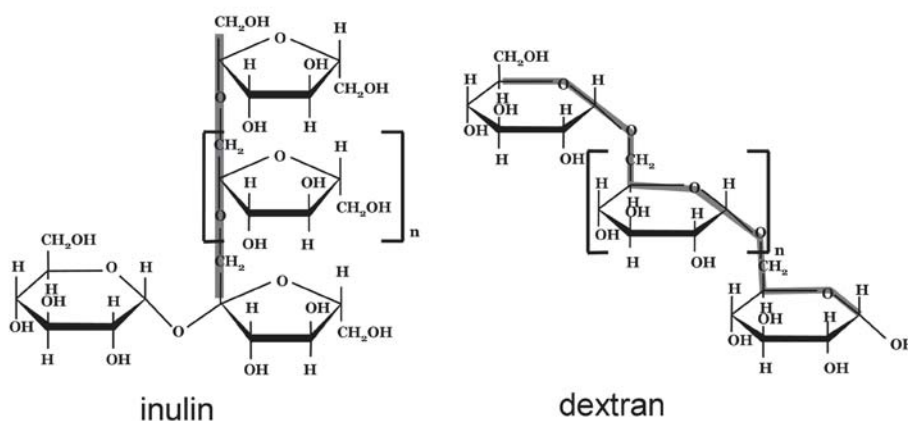


**Figure 2.1** Schematic overview of the steric hindrance of different types of sugars and their compactness of coating of proteins.

of the oligosaccharide (compare figure 2.1B and E). Continuing this train of thought, rigid oligosaccharides would not be able to accommodate to the irregular surface of the protein, whereas oligosaccharides with a flexible molecular structure would be better able to do so, resulting in a more efficient stabilization by the latter oligosaccharides.

To test this hypothesis, oligosaccharides dextran and inulin of approximately the same molecular weight (6 kDa and 4 kDa, respectively, and 70 kDa for the large oligomers) were used in this study. These molecules have different flexibilities, however. Molecular dynamics simulations have shown that inulin, which has previously been applied successfully as a stabilizer of various proteins<sup>4,20,21</sup>, is highly flexible when dissolved in water.<sup>22</sup> Hinrichs *et al.*<sup>15</sup> showed that polyethylenglycol (PEG) and inulin are compatible, while PEG and dextran are not. As a rule of thumb polymers that have similar flexibilities are compatible.<sup>23</sup> As PEG is a highly flexible molecule, the results of Hinrichs *et al.*<sup>15</sup> are in line with the simulation data and strongly indicate that inulin is more flexible than dextran. An explanation for the difference in molecular flexibility between inulin and dextran is how their backbones are constructed. Inulin is thought to have a flexible molecular structure because the backbone does not include the ring structure of the fructose units (figure 2.2).<sup>24</sup> The backbone of dextran runs through three atoms of the ring structure of each glucose unit, resulting in a more rigid backbone compared to inulin. Additionally, inulin is mostly comprised of furanose rings, which are smaller and more flexible than glucose units.<sup>22,25</sup>

Using four different-sized model proteins, we tested how size and molecular flexibility of sugars affect their ability to stabilize proteins. All proteins were freeze-dried in the presence of various sugars representing the situations in figure 2.1 and subsequently subjected to an accelerated stability test.



**Figure 2.2** Molecular structure of flexible inulin and rigid dextran. The backbone is highlighted in grey.

## MATERIALS AND METHODS

### MATERIALS

Hepatitis B surface antigen (HBsAg) was provided by the Serum Institute of India Ltd. (Pune, India). Insulin was provided by MSD (Oss, The Netherlands). A suspension of LDH from rabbit muscle in 3.2 M ammonium sulfate, dextran 6 kDa, dextran 70 kDa, bovine serum albumin (BSA), magnesium chloride, *ortho*-nitrophenyl- $\beta$ -galactoside, reduced  $\beta$ -nicotinamide adenine dinucleotide disodium salt hydrate (NADH), sodium pyruvate, potassium sodium tartrate tetrahydrate, 3,5-dinitrosalicylic acid and phenol were obtained from Sigma-Aldrich (Zwijndrecht, The Netherlands).  $\beta$ -galactosidase was obtained from Sorachim (Lausanne, Switzerland). Trehalose was obtained from Cargill (Amsterdam, The Netherlands). Lactose monohydrate was obtained from DMV-Fonterra excipients (Goch, Germany). Inulin 4 kDa was a generous gift from Sensus (Roosendaal, The Netherlands). Sodium sulfite was obtained from Spruyt Hillen BV (IJsselstein, The Netherlands). Acetonitril was obtained from Biosolve (Valkenswaard, The Netherlands). Sodium hydroxide and glucose were obtained from Merck (Darmstadt, Germany). Phosphate buffered saline (PBS) consisted of 10 mM phosphate, 137 mM sodium chloride and 2.7 mM potassium chloride (pH 7.4). Phosphate buffer for the  $\beta$ -galactosidase assay consisted of 75 mM disodium hydrogen phosphate and 25 mM potassium dihydrogen phosphate (pH 7.3). The buffer used for the LDH assay solutions was a 0.1 M solution of monopotassium phosphate adjusted to pH 7.5 with sodium hydroxide. Hepes buffer (HB) consisted of 2 mM Hepes at a pH of 7.5.

### PREPARATION OF POWDER FORMULATIONS

In this study, four different proteins were used: insulin (6 kDa), hepatitis B surface antigen (HBsAg) (a virus like particle consisting of around 100 subunits with a molecular mass of 25 kDa each<sup>26</sup>), lactate dehydrogenase (LDH) (140 kDa), and  $\beta$ -galactosidase (540 kDa). For each protein, six powder formulations were prepared by freeze-drying in the presence of either one or two sugars or no sugar at all (control). The sugars used in this study were trehalose, dextran 6 kDa, dextran 70 kDa, inulin 4 kDa or a mixture of dextran 70 kDa and trehalose (1:1 weight ratio).

First, protein solutions of 1 mg/mL in HB were prepared. The LDH suspension was dialyzed overnight at 4 °C against HB using a ThermoScientific Slide-a-lyzer cassette with a molecular weight cut-off of 7 kDa prior to dilution. Next, the sugar was dissolved upon heating at a concentration of 62.25 mg/mL in HB. After cooling the sugar solution to room temperature, the protein and sugar solution were mixed in a 1:4 v/v ratio resulting in a final protein concentration of 0.2 mg/mL and sugar concentration of 49.8 mg/mL (protein:sugar ratio = 1:249 (w/w)). Next, 200  $\mu$ L of this solution was pipetted into a 4 mL HPLC glass vial, which was then immersed into liquid nitrogen until the protein-sugar solution was frozen.



Subsequently, the vials with the frozen solutions were placed on a precooled shelf (-35 °C) of a Christ Epsilon 2-4 freeze-dryer (Salm & Kipp, Breukelen, The Netherlands). The frozen solutions were then freeze-dried at a pressure of 0.220 mBar and a condenser temperature of -85 °C for 24 hours. Thereafter, the pressure was decreased to 0.050 mBar while the shelf temperature was increased to -15 °C at a rate of 10 °C/min. This temperature was maintained for 2 hours, after which the temperature was further increased to 0 °C at a rate of 5 °C/min and lastly to 20 °C at a rate of 1 °C/min. The last part of the secondary drying was maintained for a minimum of 8 hours.

#### **DIFFERENTIAL SCANNING CALORIMETRY**

Differential Scanning Calorimetry (DSC) was used to determine the T<sub>g</sub> of the powder formulations after freeze-drying and after storage. The samples (about 2 to 3 mg) were analyzed in an open aluminum pan and placed in a Q2000 DSC (TA Instruments, Ghent, Belgium) and were preheated for 3 minutes at 80 °C to remove the residual water. Next, the sample was cooled to 20 °C and subsequently raised to 240 °C at 20 °C/min. The inflection point of the step transition in the thermograph was taken as the T<sub>g</sub>. Additionally, placebo sugar samples, containing sugar and buffer, were stored at 60 °C in open DSC pans. After 1 week, pans were hermetically sealed and measured to determine the effect of water uptake during storage on the T<sub>g</sub>. DSC settings were identical, except for the preheat step which was left out. The T<sub>g</sub> of the maximum freeze concentrated fraction, the T<sub>g</sub>', was determined by rapid cooling of approximately 40 mg of sugar solution (49.8 mg/mL in HB) to -60 °C and subsequently heating the sample to 30 °C at 20 °C/min.

#### **SUMNER ASSAY**

The amount of reducing groups in each sugar was measured by means of the Sumner assay according to the procedure described by Franssen *et al.*<sup>27</sup>. To a glass tube, 1.0 mL of an aqueous sugar solution was added. Next, 1.5 mL of aqueous Sumner assay solution was added, containing 200 mg/mL NaK-tartrate, 10 mg/mL dinitrosalicylic acid, 10 mg/mL NaOH and 2 mg/mL phenol. Finally, 100 µL of a freshly prepared 0.24 M of Na<sub>2</sub>SO<sub>3</sub> was added. The glass tube was vortexed and then placed in a waterbath at 95 °C for 15 minutes. After cooling to room temperature the absorbance was measured at 630 nm using a Unicam UV 500 spectrophotometer (ThermoSpectronic, Cambridge, UK) and compared to a calibration curve of 0.10 – 1.00 mg/mL glucose solutions. The amount of reducing groups of a sample was related to that of glucose, which was thus by definition 100% reducing.

#### **WATER CONTENT DETERMINATION**

The amount of residual moisture after freeze-drying was determined by Karl Fischer coulometric water titration using an 831 KF Coulometer (Metrohm Applikon, Schiedam, The

Netherlands). Prior to analysis, the powder was dissolved in Hydranal<sup>®</sup>-Coulomat AG (Karl Fischer reagent).

### STORAGE STABILITY TESTING

In order to evaluate the storage stability after freeze-drying, the formulations were stored at 60 °C at a relative humidity <10%. Immediately after freeze-drying and after 1, 2, and 4 weeks of storage, samples were analyzed by an assay specific for each protein: reversed phase high-performance liquid chromatography (RP-HPLC; for insulin), ELISA (for HBsAg) or by enzymatic activity assay (for  $\beta$ -galactosidase and LDH) as well as by intrinsic fluorescence spectroscopy (all proteins). The results of all analysis were compared to the result immediately after freeze-drying and expressed as a percentage of this result (*i.e.* the results immediately after freeze-drying were taken as 100%). Since changes in activity during processing were found to be small and similar for all formulations per protein (data not shown), this aspect was not further considered in this work.

### RP-HPLC

A modified gradient RP-HPLC method, based on the United States Pharmacopeia assay for insulin<sup>28</sup>, was used to determine the amount of undeamidated insulin after freeze-drying and after storage at 60 °C. Prior to analysis, the powder formulations were reconstituted in 0.01 N hydrogen chloride to a concentration of 80  $\mu$ g/mL. An Ultimate 3000 HPLC (Dionex) with a ChromSpher C18 column (3 mm x 250 mm) was used. A gradient of two mobile phases was applied. Mobile phase A consisted of 10% acetonitrile, 70% Milli-Q water and 20% Na<sub>2</sub>SO<sub>4</sub> buffer (2 M, pH 2.3) while mobile phase B consisted of 40% acetonitrile, 40% Milli-Q water and 20% Na<sub>2</sub>SO<sub>4</sub> buffer (2 M, pH 2.3). The gradient scheme was: 0 – 2 minutes, 65% mobile phase B; 2 – 16 minutes, mobile phase B gradually increased to 100%; 16 – 17 minutes, mobile phase B decreased to 65%; 17 – 27 minutes, mobile phase B kept at 65%. The flow rate was 1.0 mL/min and the column was kept at a temperature of 40 °C. For the detection of insulin a UV detector was used, set at a wavelength of 214 nm. The peak area was determined and compared to the peak areas of freshly prepared reference samples.

### HBSAG ENZYME-LINKED IMMUNOSORBENT ASSAY (ELISA)

The ability to induce an immune response is depending on the antigenicity of the vaccine, which is depending on the epitopes of the vaccine. The integrity of the epitopes on the 'a' determinant of HBsAg was investigated using a Murex HBsAg version 3 ELISA kit (Murex Biotech Limited, Dartford, UK). The powder formulations were reconstituted and diluted in PBS to a concentration of 1 ng/mL and pre-incubated for 1 hour at 37 °C in microwells coated with a mixture of mouse monoclonal antibodies specific for different epitopes on the 'a' determinant of HBsAg. Next, affinity purified goat antibodies to HBsAg, conjugated to

horseradish peroxidase, were added to the wells and these wells were incubated at 37 °C for 30 minutes. After washing, a substrate solution containing 3,3', 5,5'-tetramethylbenzidine (TMB) and hydrogen peroxidase was added. The conversion of TMB by peroxidase was stopped after 30 minutes with sulfuric acid and measured spectrophotometrically at 415 nm with a Benchmark Microplate reader (BioRad, Hercules, CA, USA). The absorbance was compared to unprocessed HBsAg, which was stored in a refrigerator during the stability study.

#### LDH ACTIVITY ASSAY

Functionality of LDH was determined by measuring its ability to convert pyruvate into lactate. At pH 7.5, LDH converts pyruvate and NADH to lactate and NAD<sup>+</sup>. NADH absorbs at 340 nm, while NAD<sup>+</sup> does not, thus allowing this reaction to be monitored spectrophotometrically. A unit of activity is defined as the conversion of 1.0  $\mu$ mole of pyruvate per minute at pH 7.5 at 37 °C. LDH containing samples were diluted to a concentration of approximately 0.25-0.025 unit/ml with a solution of 0.01% BSA in 0.1 M potassium phosphate (pH 7.5) buffer. The analysis was carried out in a flat-bottom 96-wells plate (Greiner Microlon®600 F-bottom). 50  $\mu$ L 8 mM sodium pyruvate in the aforementioned phosphate buffer was added to 100  $\mu$ L of the diluted LDH solution. The plate was then incubated at 37 °C for 10 minutes. Lastly, 50  $\mu$ L of a 1.2 mM freshly prepared NADH solution in the same buffer was added to start the reaction. The absorption at 340 nm was measured every minute for up to 1 hour using a Biotek Synergy HT multi-detection microplate reader. The reaction rate was determined from the slope of the linear part of the absorption-time curves. A correction for the slope of references without LDH was made. Reaction rates were shown to be linear to the concentration of LDH for a range from 0.5 to 0.001 unit/mL (data not shown). Because a reference solution of LDH was not stable over 4 weeks (even when stored refrigerated), activities of the samples were related to their activities directly after freeze-drying.

#### $\beta$ -GALACTOSIDASE ACTIVITY ASSAY

The structural integrity of  $\beta$ -galactosidase was evaluated using an enzymatic activity assay, based on the rate of conversion of a colorless substrate, ortho-nitrophenyl- $\beta$ -galactoside, into the yellow product, ortho-nitrophenol, by  $\beta$ -galactosidase. First, the powder formulations were reconstituted in 0.1 M phosphate buffer (pH 7.3) and diluted to a concentration of 10  $\mu$ g/mL. Next, the wells of a 96-wells plate (Greiner Microlon®600 F-bottom) were pre-incubated with 20  $\mu$ L  $\beta$ -galactosidase solution (samples at 10  $\mu$ g/mL, calibration curve: 4 – 20  $\mu$ g/mL), 230  $\mu$ L of 1.0 mM MgCl<sub>2</sub> and 0.008% bovine serum albumin (BSA) in phosphate buffer (0.1 M, pH 7.3) for 10 minutes at 37 °C. Afterwards, 20  $\mu$ L of a 34 mM *ortho*-nitrophenyl- $\beta$ -galactoside solution in phosphate buffer (pH 7.3) was pipetted into the wells and the absorption at 415 nm was measured 10 times with an interval of 30 seconds using a

Benchmark microplate reader (BioRad, Hercules, CA, USA). The absorption was plotted as a function of time and the slope of this straight line was taken as a measure of the enzymatic activity of  $\beta$ -galactosidase and was compared to unprocessed  $\beta$ -galactosidase, which was stored in a refrigerator during the stability study.

### INTRINSIC FLUORESCENCE SPECTROSCOPY

Steady state fluorescence spectroscopy measurements were performed using a QuantaMaster™ 40 spectrofluorometer (PTI, Birmingham, AL, USA) in a similar manner as reported previously.<sup>20</sup> Samples were measured in a rectangular quartz cuvette with a path length of 10 mm. The temperature was maintained constant at 20.0 °C during all measurements. Before measuring, the lyophilized samples were reconstituted with PBS to a protein concentration of 10  $\mu$ g/mL and 1.5 mL of this solution was placed in a cuvette. An excitation wavelength of 295 nm was used for LDH,  $\beta$ -galactosidase and HBsAg to specifically excite the tryptophan residues. Emission scans were performed from 300 to 400 nm. For insulin, which does not contain tryptophan residues, an excitation wavelength of 279 nm was used to excite the tyrosine residues. Emission scans were performed from 280 to 340 nm. For all scans excitation slits of 2.5 nm and emission slits of 2.5 nm were used and all scans were performed at a speed of 100 nm/s. For each sample 5 scans were performed and the result was averaged and corrected for background caused by PBS.

## RESULTS

### GLASS TRANSITION TEMPERATURE (T<sub>g</sub>)

Table 2.1 shows the T<sub>g</sub> values of the different sugars and of the different protein formulations, immediately after freeze-drying and evaporation of residual moisture (3 min preheat at 80 °C). The T<sub>g</sub> values of the various formulations did not depend on the type of protein used and were similar to values found elsewhere.<sup>15,29</sup> The highest glass transition temperature was found for dextran 70 kDa based formulations and the lowest for formulations containing trehalose. The glass transition temperature of the formulations containing both dextran 70 kDa and trehalose was about the same as the glass transition temperature of the inulin 4 kDa based formulations. The T<sub>g</sub> of dextran 6 kDa was between that of inulin 4 kDa and dextran 70 kDa. The glass transition temperature did not change during storage (data not shown). The influence of protein on the T<sub>g</sub> was negligible, as was shown by the minor differences between the T<sub>g</sub> of the protein containing powders and the T<sub>g</sub> of the corresponding sugar. The T<sub>g</sub> of the placebo samples measured in hermetically sealed pans (without preheat) after storage was much lower than the samples measured in open pans, as can be explained by the plasticizing effect of water. The T<sub>g</sub> of the larger sugars was lowered more than for the smaller sugars. Water has a T<sub>g</sub> of approximately

-109 °C<sup>30,31</sup>, the relative difference in Tgs between water and the sugars is thus larger for the larger sugars, providing a possible explanation for the larger drop in Tg. The Tg of all the samples is still 25-30 °C above the storage temperature of 60 °C for trehalose and even higher for the other samples. The Tg' values of all the formulations are higher than the shelf temperature used during primary drying and no visual collapse was observed. The moisture content of the trehalose and inulin formulations was low (<2%) and slightly higher in the formulations containing dextran (3–4%).

**Table 2.1** Tg and Tg' of pure sugars and different protein formulations (protein-sugar ratio 1:249 (w/w)), and water content of placebos immediately after freeze-drying (n=1 for Tg determination; n=3 for water content and Tg' determinations; n.d.= not determined). Formulations contained < 1% buffer on a dry substance basis.

Sugar	Glass transition temperature (°C)						Tg' (°C)	Water content (weight % ± S.D.)
	Insulin	LDH	β-galactosidase	HBsAg	Pure sugar	Pure sugar (closed pan after storage)	Pure sugar solution	Pure sugar (after freeze drying)
Trehalose	121	122	121	121	122	88	-27.9±0.7	1.8±0.1
Dextran 70 kDa + trehalose (1:1)	159	159	158	159	159	110	-19.7±0.8	3.0±0.1
Dextran 70 kDa	223	224	224	223	224	167	-11.2±0.4	4.1±0.3
Dextran 6 kDa	192	190	192	190	193	144	-14.1±0.2	4.2±0.2
Inulin 4 kDa	154	155	154	155	156	119	-16.9±0.9	1.1±0.2
Lactose	n.d.	n.d.	n.d.	n.d.	119	92	n.d.	n.d.
Trehalose + lactose (4:1)	n.d.	n.d.	n.d.	n.d.	121	89	n.d.	n.d.

## REDUCING GROUPS

The Sumner assay was performed to determine the amount of reducing groups in each sugar. Reducing groups of the sugar can react with amino acids in the protein. Table 2.2 shows that trehalose is a non-reducing sugar, oligosaccharides inulin 4 kDa and dextran 6 kDa contained some reducing groups, and polysaccharide dextran 70 kDa contained little reducing groups. Dextran is a non-reducing sugar, apart from one glucose unit at the end of the chain, which can form a reducing group by ring opening.<sup>32</sup> For the smaller dextran, these end groups are relatively more abundant compared to the larger dextran, explaining the difference in amount of reducing groups found. In theory, inulin should not contain any reducing groups because of the way the fructose and glucose groups are linked.<sup>21</sup> However,

**Table 2.2** Percentage of reducing groups relative to glucose as determined by Sumner assay (n=3)

Sugar	Percentage reducing groups relative to glucose
Trehalose	0.1 (±0.0)
Dextran 70 kDa	0.6 (±0.0)
Dextran 6 kDa	10.9 (±0.8)
Inulin 4 kDa	5.0 (±0.1)
Lactose	82.1 (±3.1)

if the glucose end group of inulin is cleaved, a reducing fructose group at the end of the chain as well as a reducing glucose are created. This could explain the amount of reducing groups found by the Sumner assay, which were similar to previously reported values.<sup>21</sup> Even though dextran and inulin contain some reducing groups, no Maillard browning was observed during storage at 60 °C. In fact, even for proteins freeze-dried in the presence of lactose, which contains a large number of reducing groups, as was confirmed by our test (table 2.2), no browning was detected. This may be explained by the near absence of water present in the various samples. It should however also be noted that the first stages of the Maillard reaction occur without browning.

#### STORAGE STABILITY

To investigate the stability of the four proteins after freeze-drying in the presence of the various sugars, all formulations were stored at 60 °C and analyzed after 1, 2, and 4 weeks of storage and compared to the result immediately after freeze-drying. Samples were analyzed using an assay specific for each protein and by fluorescence spectroscopy.

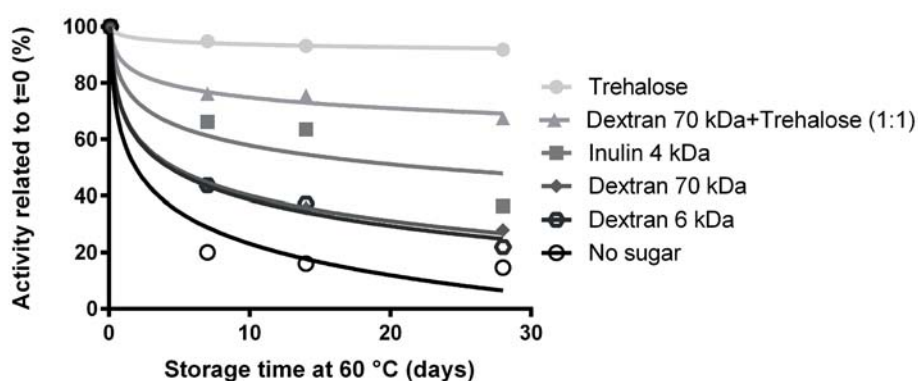
#### Protein specific assays

Figure 2.3 shows an example of the activity versus time profile of the formulations containing LDH and the various sugars. The other proteins show similar, but numerically different profiles, their activity versus time profiles can be found in the supporting information. Standard deviations of the assays were generally smaller than 5% and were not shown for clarity reasons. To obtain an overview of the data, a kinetic fit of the decrease of protein that remained intact during storage at 60 °C was made. As protein degradation can occur through many pathways, there was no obvious kinetic fit to be used. The function that provided the highest correlation coefficients (R) (equation 2.1), a logarithmic function, was therefore used. A square root of time based function, as used in other studies, also seemed consistent with the data, but yielded lower R-values. R-Values were generally larger than 0.95 and always larger than 0.90.

$$(2.1) \quad F(t) = -A \ln(t) + B$$

where  $F(t)$  is the amount of protein still intact, relative to the initial activity,  $t$  time in days and the fitting factor  $A$  provides information about the rate of degradation of the specific formulation.  $B$  is a fitting value that completes the equation and is not used further. Figure 2.4 depicts the degradation rate constants and the accompanying correlation coefficients. Since a logarithmic fit cannot incorporate  $t=0$ ,  $t=0.01$  was used for the fits.

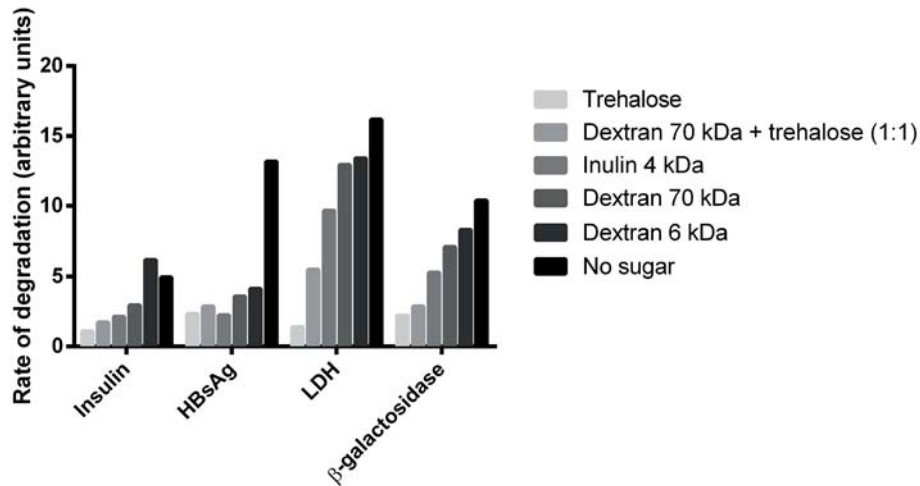
For insulin, LDH, and  $\beta$ -galactosidase, it was found that the rate of degradation was lowest when trehalose was used as a stabilizer. HBsAg was best stabilized by inulin 4 kDa. However, the differences between stabilizing capacities of trehalose and inulin 4 kDa were small. Furthermore, it was found that all four proteins were more stable when freeze-dried in the presence of the inulin 4 kDa than when freeze-dried in the presence of the dextran 6 kDa. Remarkably, the insulin formulation with dextran 6 kDa was even less stable than the formulation of insulin only (no sugar). Proteins freeze-dried in the presence of dextran 70 kDa were more stable than when the smaller dextran was used. The stabilizing capacity of dextran 70 kDa was substantially increased when it was mixed in a 1:1 weight ratio with trehalose.



**Figure 2.3** Storage stability of LDH formulations by activity assay analysis after 1, 2, and 4 weeks of storage at 60 °C. The results are normalized to day 0 (immediately after freeze-drying). ( $n=1$  per time point; samples were measured in triplicate. The relative standard deviation was between 0 and 3%, error bars are not shown for clarity purposes.)

#### Intrinsic fluorescence spectroscopy

To monitor irreversible changes in the conformation of the proteins during storage, intrinsic fluorescence spectroscopy was used. Conformational changes lead to changes in the local environment of tryptophan or tyrosine (in the case of insulin) residues, which influences the fluorescence intensity of these residues. Additionally, formation of insoluble aggregates could also influence the fluorescence intensity. Any changes in the fluorescence intensity



**Figure 2.4** Rate of degradation of the proteins during storage for the various formulations (fitting factor A from equation 2.1).

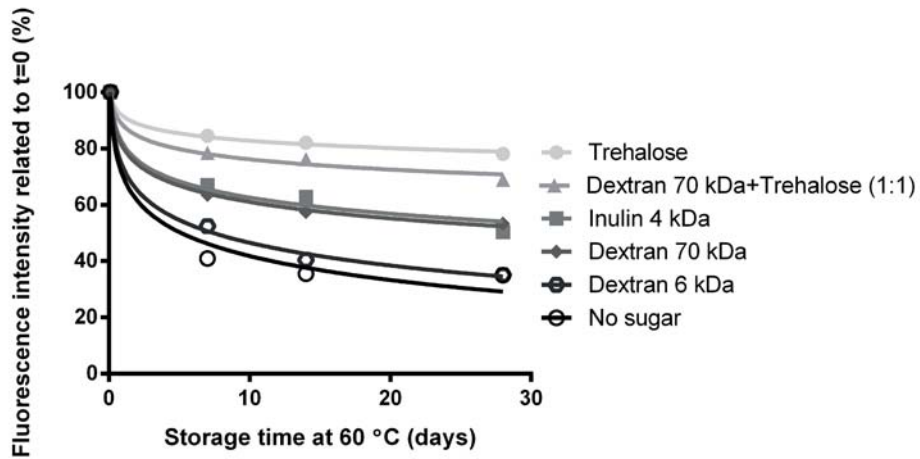
are thus undesired. As with any protein analysis after reconstitution, fluorescence only measures irreversible changes during storage as some changes could have reverted with reconstitution.

Figure 2.5 shows the changes in maximum fluorescence intensity of the formulations containing LDH and the various sugars. The other proteins show similar, but numerically different profiles; their fluorescence intensity versus time profiles can be found in the supporting information. Similar to the protein specific assays, a logarithmic function (equation 2.1) was used to fit the rate of change in tryptophan fluorescence intensity over time. Fitting factors ( $R$ ) were at least 0.90 except for the combination of insulin and trehalose ( $R = 0.756$ ). Here too, the fitting parameter ( $A$ ) from equation 2.1 was taken as a measure for the degradation rate for comparison of the different formulations.

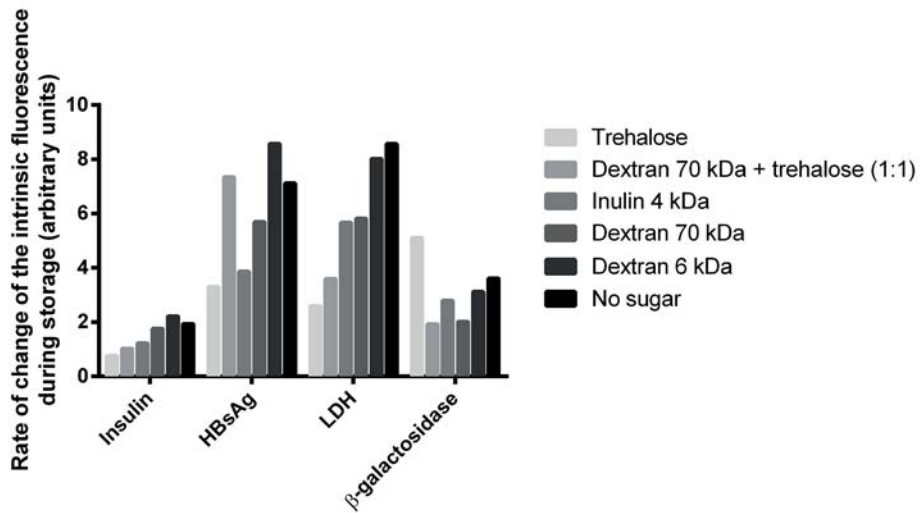
Figure 2.6 shows the same trends as found with the protein specific assays. Proteins freeze-dried in the presence of trehalose showed the least change in maximum fluorescence intensity, except for  $\beta$ -galactosidase. For  $\beta$ -galactosidase, the most stable formulation was achieved when using a combination of dextran and trehalose, yet it should be noted that the differences between the various formulations were relatively small for this protein. When both oligosaccharides (molecularly rigid dextran 6 kDa and flexible inulin 4 kDa) are compared, the changes in conformation were for every protein smaller when they were freeze-dried in the presence of inulin 4 kDa. Conformational changes in the structure of insulin and HBsAg freeze-dried in the presence of dextran 6 kDa were even greater during storage than when no sugar was used. Proteins freeze-dried in the presence of dextran 70 kDa yielded products



that were more stable than proteins freeze-dried in the presence of dextran 6 kDa but not as stable as when inulin 4 kDa was used. Furthermore, with the exception of HBsAg, the decrease in maximum fluorescence intensity was small when a mixture of dextran 70 kDa and trehalose was used, compared to the formulations containing dextran 70 kDa only.



**Figure 2.5** Maximum fluorescence intensity of LDH formulations after 1, 2, and 4 weeks of storage at 60°C. The results are normalized to day 0 (immediately after freeze-drying). (n=1 per timepoint, result is average of 5 scans)



**Figure 2.6** Rate of change (fitting factor A from equation 2.1) of the intrinsic tryptophan (or tyrosine for insulin) fluorescence of the proteins during storage for the various formulations.

### Degradation by reducing sugars

The Sumner assay indicated that the amount of reducing groups differed for the different sugars. Even though no browning was observed upon storage, it was important to rule out the effect of reducing groups on stability, before making any statements about the influence of sugar size and molecular flexibility on their ability to stabilize proteins. Therefore stability tests with proteins LDH and  $\beta$ -galactosidase were carried out to assess the effect of presence of reducing sugars. These stability tests were carried out with the same protein, but at a later date. The obtained degradation rates were not the same as the initial test, but this did not influence the conclusions drawn from these experiments, as these were based on the relative differences between the reducing formulations. Table 2.3 shows the results of the stability test with reducing sugars.

Lactose has the same molecular weight and a similar glass transition temperature as trehalose, but unlike trehalose it is a reducing sugar. Trehalose, lactose and the mixture of the two provided very similar results. Because the changes in fluorescence intensity were relatively small for  $\beta$ -galactosidase, R values are relatively low. The relative differences between the formulations, however, remain unaffected. There is no clear relationship between the amount of reducing groups and the rates of degradation. We can thus rule out the effect of reducing groups on storage stability of the proteins when comparing formulations in these experiments.

**Table 2.3** Rate of degradation and rate of change of intrinsic fluorescence intensity of the proteins (fitting factor A from equation 2.1) with various reducing sugars during storage with the corresponding correlation coefficient (R) of the fit (shown in parentheses) and amount of reducing groups of the sugar as determined by the Sumner assay.

Sugar	Activity assay		Intrinsic tryptophan fluorescence		Reducing groups relative to glucose
	LDH (140 kDa)	$\beta$ -galactosidase (540 kDa)	LDH (140 kDa)	$\beta$ -galactosidase (540 kDa)	
Trehalose	6.10 (0.948)	11.33 (0.851)	4.28 (0.864)	1.54 (0.355)	0.1% ( $\pm$ 0.0)
Trehalose + lactose (4:1)	6.12 (0.947)	12.62 (0.871)	4.43 (0.888)	1.02 (0.227)	16.5% *
Lactose	6.18 (0.952)	12.39 (0.824)	4.19 (0.913)	0.96 (0.222)	82.1% ( $\pm$ 3.1)

\* is based on calculation

## DISCUSSION

In this study, we tested the influence of the size and molecular flexibility of sugars on their protein stabilizing abilities. It was hypothesized that small sugars (disaccharides) were hardly sterically hindered in interacting with proteins and that for oligosaccharides the quality of the interaction between the sugar and protein depended on molecular flexibility. Proteins freeze-dried in the presence of the smallest sugar (the disaccharide trehalose) were best preserved, in regard to functionality as well as the change in conformation during storage. Furthermore, it was confirmed that the flexible oligosaccharide (inulin) indeed stabilized the proteins better than the rigid oligosaccharide (dextran).

From the degradation rates ( $A$ , from equation 2.1 for change in either activity or fluorescence) of the different formulations, shown in figures 2.4 and 2.6, it is possible to calculate the relative improvement stability of that formulation compared to the unstabilized protein, using equation 2.2.

$$(2.2) \quad \text{Relative improvement of stability} = (1 - (A_{\text{formulation}} / A_{\text{no sugar}})) \times 100\%$$

The protein without any sugar is the negative control and by definition has 0% improved stability. A perfectly stabilized formulation, would have no change in activity during storage, and thus a degradation rate ( $A_{\text{formulation}}$ ) of 0, and with that a 100% improved stability compared to the formulation without sugar.

In table 2.4 the rank order of the stabilizing capacity of the sugars for each protein group is shown, based on the relative improvement of stability from both the protein specific assays and intrinsic fluorescence spectroscopy. The formulation with the highest relative improvement of stability was ranked 1, the one with the lowest improvement was ranked lowest. In almost all cases, proteins were most stable when freeze-dried in the presence of trehalose, followed by the formulations containing a mixture of dextran 70 kDa and trehalose. This result is independent of the analytical tool used and is roughly independent of the protein and protein size used.

Furthermore, molecularly flexible inulin 4 kDa based formulations were more stable than formulations based on rigid dextran 6 kDa and 70 kDa, according to the protein specific assays as well as intrinsic fluorescence spectroscopy results. Formulations containing dextran 6 kDa or no sugar at all were least stable. Interestingly, the trends between the sugars are the same for all the proteins despite the large differences in their size (i.e. from 6 kDa for insulin to 540 kDa for  $\beta$ -galactosidase) and nature.

As described and illustrated in the introduction, small molecules like disaccharides should be able to interact closely with the protein's surface irrespective of its irregular nature. Larger molecules are less capable of doing so because of steric hindrance. A disadvantage of using

**Table 2.4** Rank order of the stabilizing capacity of the sugars for each protein and median rank of sugar over all four proteins according to the proteins specific assay and intrinsic fluorescence spectroscopy. The rank is based on the relative improvement of stability, calculated using equation 2.2 and rates from figures 2.4 and 2.6 (shown in parentheses). The formulation with the smallest improvement of stability was given the lowest rank, and vice versa.

<b>Protein Specific Assay</b>						
<b>Sugar</b>	<b>Insulin (6 kDa)</b>	<b>HBsAg (25 kDa)</b>	<b>LDH (140 kDa)</b>	<b><math>\beta</math>-galactosidase (540 kDa)</b>	<b>Median rank</b>	<b>Figure 2.1</b>
Trehalose	1 (78%)	2 (82%)	1 (91%)	1 (79%)	1	A
Dextran 70 kDa + trehalose (1:1)	2 (65%)	3 (78%)	2 (66%)	2 (72%)	2	C
Inulin 4 kDa	3 (57%)	1 (83%)	3 (40%)	3 (49%)	3	B
Dextran 70 kDa	4 (41%)	4 (73%)	4 (20%)	4 (32%)	4	D
Dextran 6 kDa	6 (-25%)	5 (69%)	5 (17%)	5 (20%)	5	E
No sugar	5 (0%)	6 (0%)	6 (0%)	6 (0%)	6	F
<b>Intrinsic Fluorescence Spectroscopy</b>						
Trehalose	1 (61%)	1 (54%)	1 (70%)	3 (42%)	1	A
Dextran 70 kDa + trehalose (1:1)	2 (47%)	5 (-3%)	2 (58%)	1 (47%)	2	C
Inulin 4 kDa	3 (36%)	2 (46%)	3 (34%)	4 (22%)	3	B
Dextran 70 kDa	4 (9%)	3 (20%)	4 (32%)	2 (44%)	3.5	D
Dextran 6 kDa	6 (-15%)	6 (-21%)	5 (6%)	5 (15%)	5.5	E
No sugar	5 (0%)	4 (0%)	6 (0%)	6 (0%)	5.5	F

disaccharides, however, is their relatively low Tg. The visual appearance of the trehalose formulation did not change (no collapsed cake), which suggests that the Tg was well above 60 °C. To verify this observation, the Tg of placebo samples in hermetically sealed pans were measured after a week of storage at 60 °C, thus showing the actual Tg including moisture at that time. It was found that the Tg was still 25-30 °C above the storage temperature for the disaccharides and even more for the other sugars. As shown by Hancock *et al.*<sup>33</sup> there is still significant molecular mobility at temperatures above the Kauzmann temperature, which is estimated to be roughly 50 °C below the Tg. Recent work of Grasmeyer *et al.*<sup>4</sup> also showed that when the Tg of a sample is increased past 10 to 20 °C above the storage temperature, the stabilization mechanism that limits stability shifts from vitrification to water replacement (e.g. interaction between protein and sugar). This is in line with findings of Mizuno and Pikal, which show that proteins in the solid state display a transition in global protein mobility at temperatures well below the Tg.<sup>34</sup> Storage at higher relative humidities would further suppress the temperature at which these motions would occur, potentially critically lowering the Tgs of the disaccharide samples below this threshold. In that respect, sugars with a higher Tg (like oligosaccharides) are preferred.

Comparing both oligosaccharides in this study showed that proteins freeze-dried in the presence of the molecularly flexible inulin 4 kDa are far better stabilized than proteins freeze-dried in the presence of rigid dextran 6 kDa, even though the Tg of dextran 6 kDa is substantially higher than the Tg of inulin 4 kDa; for the dry sugars 192 °C versus 154 °C, respectively. Even though the molecular weights of the oligosaccharides are not identical, the differences in their stabilizing capacities are much larger than can be explained by size alone. As described in section *Degradation by reducing sugars*, the influence of the Maillard reaction was ruled out for these results.

Clearly, protein stabilization by sugars is not depending on the Tg of the sugar alone. This is illustrated by trehalose, which under these circumstances performed best, whilst having the lowest Tg of the used formulations. If vitrification of the sample is sufficient, the ability of a sugar to replace or maintain hydrogen bonds of the protein during drying, and with that its ability to minimize protein mobility, becomes the limiting factor for stabilization.<sup>4</sup> This translates to a close contact of the amorphous sugar with the irregular surface of the protein as described in the introduction. We hypothesized that flexibility of the backbone of oligosaccharides determines whether or not the sugar can do so. As expected, the more flexible inulin stabilized the proteins better during storage than the similarly sized rigid dextran, presumably by the better and more complete interaction at the surface of the protein.

Using in-line near-infrared spectroscopy during freeze-drying, it was shown that these protein-sugar interactions are formed during drying and that increasing amounts of stabilizers resulted in increasing amounts of both interactions and stabilization.<sup>35</sup> It is therefore most likely that the differentiation between the amount of interactions occurs mainly during drying, where the smaller and molecularly more flexible sugars form more tight interactions than their larger and more rigid counterparts.

Such protein-sugar interactions are not only needed for good protein stabilization but are also in a way necessary to prevent phase separation of the protein and sugar. Phase separation basically makes any sugar-protein interaction impossible. The size of the oligomers and proteins implies that the excluded volumes of these compounds are relatively large. For that reason the entropy of mixing is relatively low, implicating that the enthalpy rise caused by sugar-protein interaction should be low or preferably negative to avoid phase separation.<sup>36</sup> It might therefore be useful to investigate the miscibility of these or similar protein-sugar systems to further elucidate the mechanism by which various types of sugars stabilize proteins.

Proteins freeze-dried in the presence of a rigid oligosaccharide (dextran 6 kDa) were less stable than when a rigid polysaccharide (dextran 70 kDa) was used. Since dextran 6 kDa is a lot smaller than dextran 70 kDa, it was expected that proteins would be better stabilized by the smaller dextran. Again, the number of reducing groups could not provide an

explanation for this result. A possible explanation for the observed difference could lie in the branched structure of dextran, which is most likely more pronounced in the larger dextran. Potentially the different branches could allow for some accommodation to the surface of the protein. The low stabilizing effect of dextran 70 kDa compared to trehalose can be ascribed to the large and bulky nature of dextran 70 kDa.

Addition of trehalose to dextran 70 kDa greatly improved the stability of the proteins. The T<sub>g</sub> of this formulation was higher than that of the formulation containing only trehalose. We hypothesize that the molecular mixture of trehalose and dextran combines the interaction capacity of trehalose with an increased T<sub>g</sub> of dextran 70 kDa. Because of steric hindrance, cavities in the coating at the surface of the protein can appear when a large and bulky stabilizer like dextran 70 kDa alone is used. Trehalose limits this steric hindrance, resulting in a more complete and compact coating and a high glass transition temperature of the final sample. The finding that trehalose alone performs better than the combination suggests that there is still some sterical hindrance of dextran 70 kDa which trehalose cannot overcome.

Our results show that the small disaccharide trehalose is the best stabilizer for proteins during storage at 60 °C for four weeks. It was illustrated that moisture can strongly lower the T<sub>g</sub> of the formulations. Here the relative humidity was below 10% and vitrification was not the limiting factor for stability. In situations where protein formulations are exposed to higher relative humidities, increased water uptake may occur, resulting in a further increase in mobility. This may be critical when the T<sub>g</sub> of the formulation is no longer significantly higher than the storage temperature, as the system is then no longer fully vitrified. For these situations, sugars with a higher T<sub>g</sub> are required to obtain an optimally stabilized product. In these cases, oligosaccharides could provide a solution in two different ways. Firstly, a flexible oligosaccharide could be used to increase the T<sub>g</sub> of the formulation, while still achieving an efficient coating of the protein or secondly, one could add an oligo- or polysaccharide to a formulation of smaller sugars. By varying the ratio between large and small sugars, a tailor-made adjustment of the T<sub>g</sub> in combination with a maximization of the compactness of the coating of the protein by the sugars would be feasible.

This study confirms our mechanistic hypothesis that size and molecular flexibility of sugars affect their ability to stabilize proteins. As long as they maintain vitrification, smaller and molecularly more flexible sugars are less affected by steric hindrance and thus better capable at stabilizing proteins. Since the four model proteins showed the same trends, in spite of their difference in size and nature, it is likely that the conclusions from this study will be applicable broadly for other proteins as well.

## **ACKNOWLEDGEMENTS**

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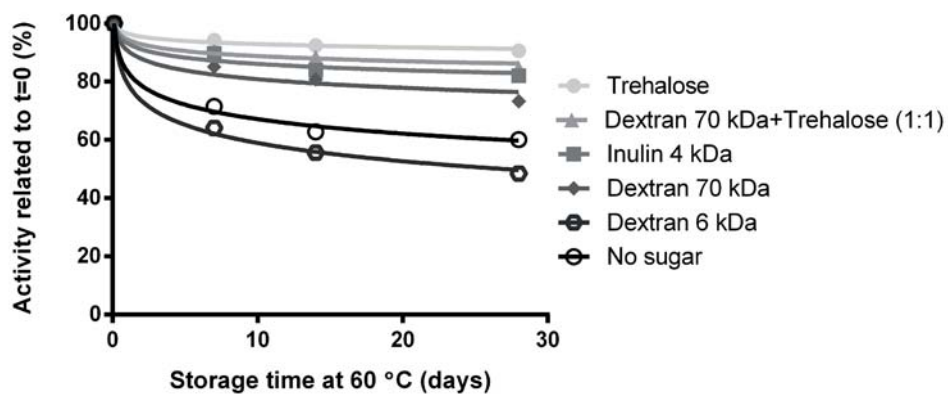


## CHAPTER 2

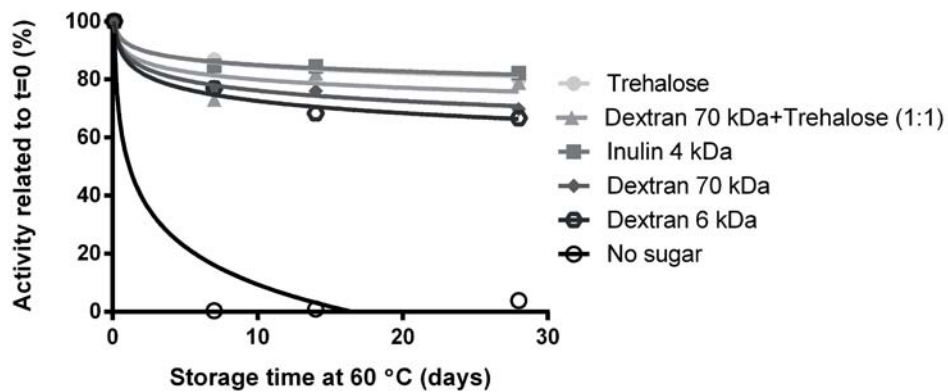
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## SUPPORTING INFORMATION

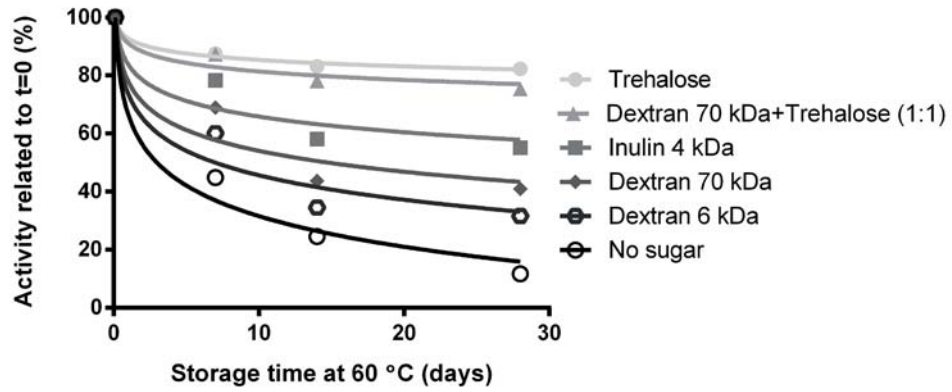
The figures below show the activity versus time profiles of insulin (figure 2.S1), HBsAg (figure 2.S2) and  $\beta$ -galactosidase (figure 2.S3) during storage. They are similar to figure 2.3 and support the information discussed in section *Protein specific assays*.



**Figure 2.S1** Storage stability of insulin formulations by activity assay analysis after 1, 2, and 4 weeks of storage at 60 °C. The results are normalized to day 0 (immediately after freeze-drying). (n=1 per time point; samples were measured in triplicate. Error bars are not shown for clarity purposes.)

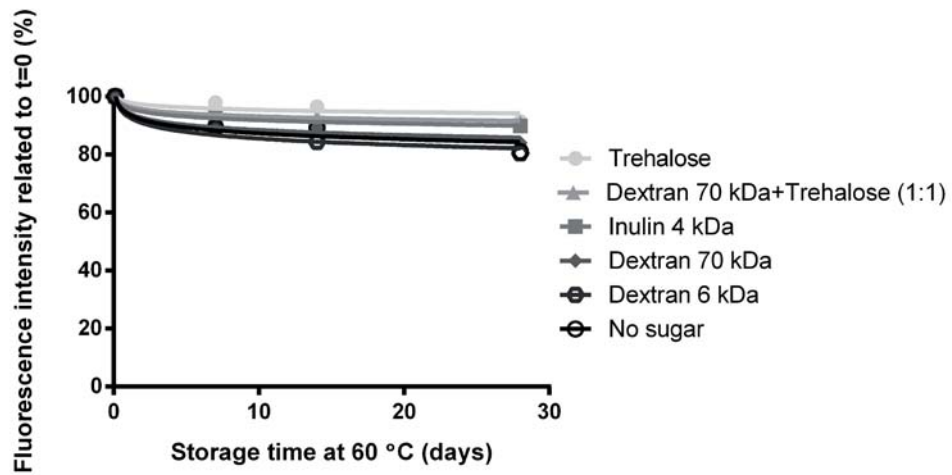


**Figure 2.S2** Storage stability of HBsAg formulations by activity assay analysis after 1, 2, and 4 weeks of storage at 60 °C. The results are normalized to day 0 (immediately after freeze-drying). (n=1 per time point; samples were measured in triplicate. Error bars are not shown for clarity purposes.)

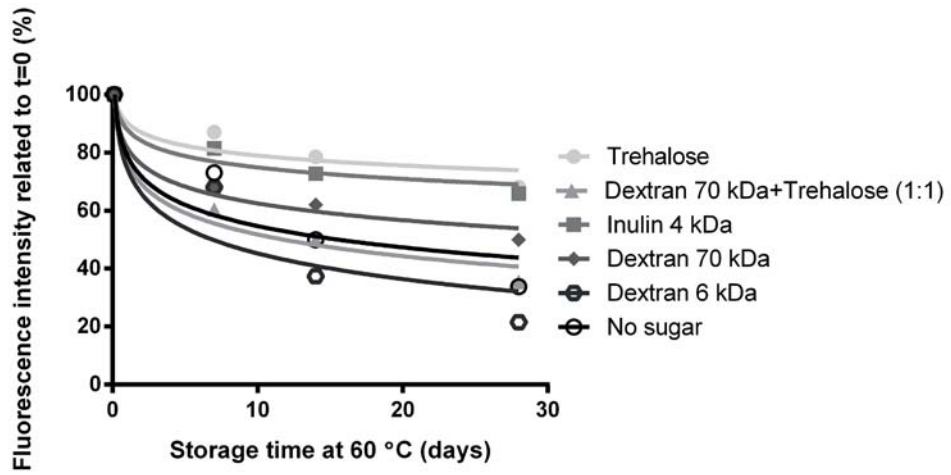


**Figure 2.S3** Storage stability of  $\beta$ -galactosidase formulations by activity assay analysis after 1, 2, and 4 weeks of storage at 60 °C. The results are normalized to day 0 (immediately after freeze-drying). (n=1 per time point; samples were measured in triplicate. Error bars are not shown for clarity purposes.)

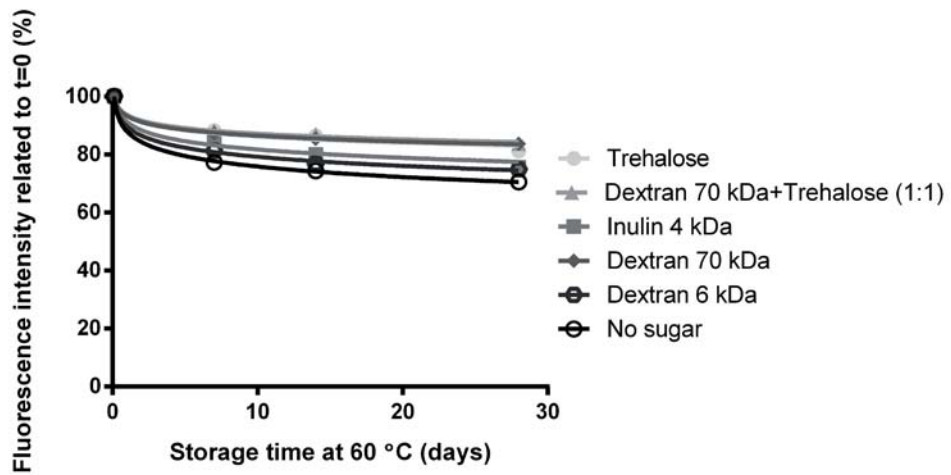
The figures below show the fluorescence intensity versus time profiles of insulin (figure 2.S4), HBsAg (figure 2.S5)en  $\beta$ -galactosidase(figure 2.S6) during storage. They are similar to figure 2.5 and support the information discussed in section *Intrinsic fluorescence spectroscopy*.



**Figure 2.S4** Maximum fluorescence intensity of insulin formulations after 1, 2, and 4 weeks of storage at 60 °C. The results are normalized to day 0 (immediately after freeze-drying). (n=1 per timepoint, result is average of 5 scans)



**Figure 2.55** Maximum fluorescence intensity of HBsAg formulations after 1, 2, and 4 weeks of storage at 60 °C. The results are normalized to day 0 (immediately after freeze-drying). (n=1 per timepoint, result is average of 5 scans)



**Figure 2.56** Maximum fluorescence intensity of  $\beta$ -galactosidase formulations after 1, 2, and 4 weeks of storage at 60 °C. The results are normalized to day 0 (immediately after freeze-drying). (n=1 per timepoint, result is average of 5 scans)

