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

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Abstract

Sugars are often used as stabilizers of protein formulations during freeze-drying. However, not all sugars are equally suitable for this purpose. Using in-line near-infrared spectroscopy during freeze-drying, it is here shown here that hydrogen bond formation during freeze-drying, under secondary drying conditions in particular, can be related to the preservation of

the functionality and structure of proteins during storage. The disaccharide trehalose was best capable of forming hydrogen bonds with the model protein, lactate dehydrogenase, thereby stabilizing it, followed by the molecularly flexible oligosaccharide inulin 4 kDa. The molecularly rigid oligo- and polysaccharides dextran 5 kDa and 70 kDa, respectively, formed the least amount of hydrogen bonds and provided least stabilization of the protein. It is concluded that smaller and molecularly more flexible sugars are less affected by steric hindrance, allowing them to form more hydrogen bonds with the protein, thereby stabilizing it better.

Keywords

Near-infrared (NIR) spectroscopy, water-replacement, vitrification, molecular flexibility, solid-state stability, Fourier transform infrared (FTIR) spectroscopy

1. Introduction

Over the past decades protein drugs have gradually grown to become important players in the pharmacological treatment of diseases. In fact, there are seven biopharmaceuticals among the ten top-selling drugs of 2014 (King, 2015). Proteins as such are not stable in solution and require refrigerated storage and transport, the so-called cold chain, to limit loss of functionality and formation of immunogenic degradation products (Chi et al., 2003). Cold chain handling is expensive and often impractical, creating serious logistical problems particularly in tropical developing countries. Therefore, protein formulations are frequently dried e.g. by spray-drying or freeze-drying (lyophilization), to create a powder that is less sensitive to degradation and does not require a cold chain (Carpenter et al., 1997; Wang, 2000). During these drying processes, however, proteins are subjected to several types of stresses, including thermal and dehydration stresses (Abdul-Fattah et al., 2007; Crowe et al.,

1990). To protect the proteins against these and storage stresses, stabilizers are required. For this purpose, small sugars (e.g. disaccharides) are often used.

Currently, there are two predominant theories regarding how lyoprotectants stabilize proteins, namely the vitrification and the water replacement theories (Arakawa et al., 1991; Chang and Pikal, 2009; Crowe et al., 1998). Vitrification theory states that protein stabilization is achieved by the formation of a glass, in which mobility is reduced so strongly that molecular mobility needed for degradation does not take place on the timescale of storage (Hancock et al., 1995). A characteristic of glasses is the glass transition temperature (Tg), above which molecular mobility increases dramatically, with potentially detrimental effects on protein stability. Therefore, glassy (amorphous) formulations should not be subjected to temperatures above their Tg (Duddu and Dal Monte, 1997; Imamura et al., 2009). Water replacement theory encompasses the idea that the sugar molecules replace the hydrogen bonds of water with the protein during drying and thus stabilize the protein conformation (Carpenter and Crowe, 1989). These two theories are not mutually exclusive; both mechanisms play a role in protein stabilization (Grasmeijer et al., 2013; Randolph, 1997). Which mechanism is prevalent depends on several factors like formulation (e.g. type of stabilizer), residual moisture, presence of plasticizers and storage temperature (Grasmeijer et al., 2013). Protein stability has also been related to fast β-relaxation in the solid of these proteins (Cicerone and Douglas, 2012). This could explain how water replacement and vitrification together result in protein stabilization by reduction of the detrimental protein mobility in the solid state.

The above-presented theories lead to specific predictions about the behavior and limitations of various sugar types. Frequently used disaccharides (sucrose and trehalose) are characterized by relatively low Tg values (Mensink et al., 2015). This means that plasticizers (e.g. residual water, atmospheric water and buffers), which lower the Tg, can critically increase molecular mobility with detrimental consequences for protein stability (Allison et al.,

2000; Duddu and Dal Monte, 1997; Lückel et al., 1997). Oligosaccharides, on the other hand, have higher Tgs, limiting their susceptibility to this problem (Allison et al., 2000; Hinrichs et al., 2001). Their size, however, imposes the problem of steric hindrance, potentially limiting their capacity to hydrogen bond with the protein (Allison et al., 1999; Tanaka et al., 1991; Taylor, 1998). Thus, in general, small sugars (e.g. disaccharides) are not ideal in the light of the vitrification theory and larger sugars (e.g. oligo- and polysaccharides) have their limitations in relation to the water replacement theory.

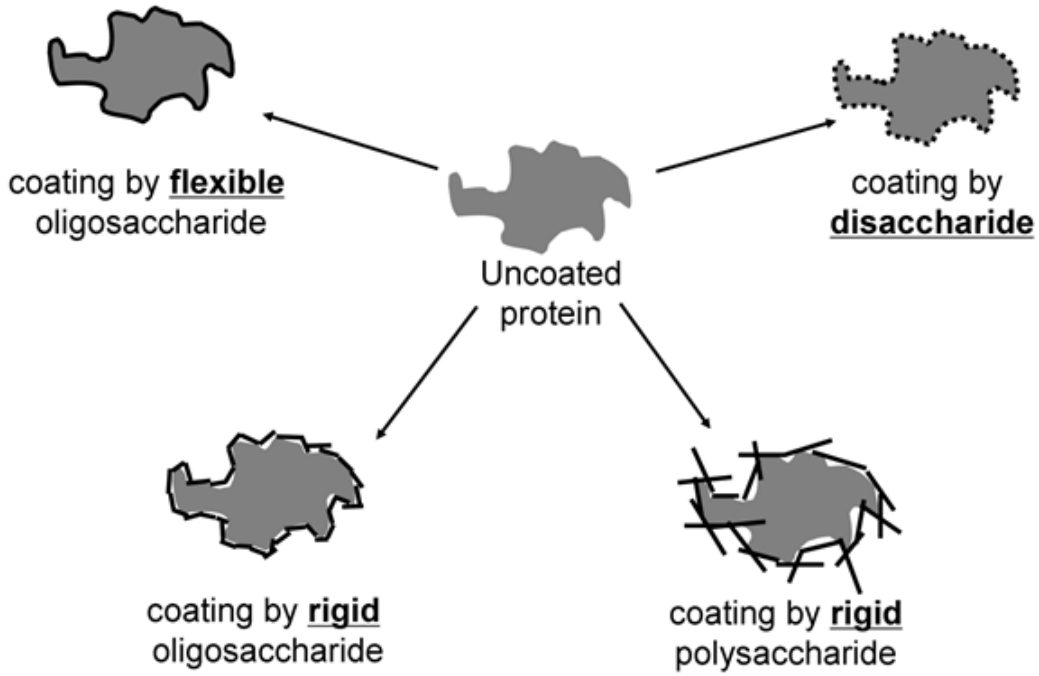


Fig 1. Schematic overview of the compactness of coating of proteins by different types of sugars (Tonnis et al., 2015). Modified and reprinted with permission from American Chemical Society.

Recent work confirmed that smaller sugars stabilize proteins better than larger sugars (Tonnis et al., 2015). In addition, it was shown that the molecularly flexible oligosaccharide inulin stabilized four model proteins better than the more molecularly rigid oligosaccharide dextran. The authors hypothesized that molecular flexibility can reduce the steric hindrance

87 associated with the relatively large size of oligosaccharides. The molecular flexibility allows
88 the sugars to accommodate to the protein structure, forming a tight coating around it, as
89 illustrated in Fig. 1. This tight coating should allow the molecularly flexible oligosaccharides
90 to form more hydrogen bonds with the protein than do molecularly rigid oligosaccharides,
91 overcoming the main limitations of oligosaccharides (Tonnis et al., 2015). Unfortunately,
92 mechanistic evidence supporting this flexibility hypothesis is still lacking. A new in-line near-
93 infrared (NIR) spectroscopy method allows monitoring of hydrogen bonding between proteins
94 and lyoprotectants (e.g. sugars) *during* lyophilization, and is therefore very suitable to test this
95 flexibility hypothesis (Pieters et al., 2012).

96 In short, the method uses a non-contact NIR probe to monitor water elimination and the
97 amide A/II band (near 4850 cm^{-1}), indicative of protein-excipient hydrogen bonding (Pieters
98 et al., 2012). Correlating these two parameters throughout the drying process allows one to
99 see which sugars effectively take over the hydrogen bonds from water during drying and
100 which do not. Based on the flexibility hypothesis, it is expected that small sugars and
101 molecularly flexible oligosaccharides form hydrogen bonds more efficiently than their larger
102 and more molecularly rigid counterparts, thereby stabilizing the proteins better. In this paper
103 mechanistic evidence explaining why size and molecular flexibility determine storage
104 stability of lyophilized proteins is provided.

105

106 ## 2. Materials & Methods

107 ### 2.1 Materials

108 The model protein used in this study, L-Lactic Dehydrogenase (LDH) from rabbit muscle,
109 was obtained as a lyophilized powder from Sigma-Aldrich (Zwijndrecht, The Netherlands).
110 The chemicals required for the activity assay of LDH (sodium pyruvate, a reduced disodium

salt hydrate of β-nicotinamide adenine dinucleotide (NADH) and bovine serum albumin (BSA)) were also purchased there. Inulin 4 kDa was a generous gift from Sensus (Roosendaal, The Netherlands). Trehalose was obtained from Cargill (Amsterdam, The Netherlands) and dextran 5 kDa and 70 kDa from Pharmacosmos (Holbaek, Denmark). Lastly the buffer components, HEPES free acid and sodium phosphate (monobasic as a dihydrate, and dibasic as a dodecahydrate) were supplied by MP Biomedicals (Illkirch, France) and Merck (Darmstadt, Germany), respectively.

2.2 Sample preparation

Solutions containing 100 mg/g of sugar in 2 mM HEPES buffer (pH 7.5) were produced. Subsequently, LDH was weighed and dissolved in the appropriate amount of this stabilizer solution to achieve a protein concentration of 10 mg/g. The protein:sugar ratio was thus 1:10 (w/w). For inulin, the stabilizer solution was mildly heated to achieve complete dissolution, and the solution was allowed to cool again before addition of the protein. For the NIR-monitored lyophilization runs, 1.5 mL of this solution was placed in 4 mL vials of type 2R (type I glass, Fiolax clear, Schott, Müllheim, Germany) with corresponding lyophilization stoppers (West, Eschweiler, Germany). The samples for stability testing and Fourier transform infrared spectroscopy (FTIR) measurements were also lyophilized in 2R type 4 mL vials, yet in 200 µL aliquots. The smaller fill volume is assumed to cause faster drying, but not influence the storage stability. Also solutions with different protein-sugar ratios were prepared and lyophilized as described above. The combined concentration of LDH and sugar of these solutions was 50 mg/mL in 2 mM HEPES buffer (pH 7.5). Protein-sugar ratios ranged from 1:7 to 7:1 (w/w), each ratio was produced in duplicate and those were measured in triplicate.

2.3 Lyophilization

Samples were lyophilized using an Amsco FINN-AQUA GT4 freeze-dryer (GEA, Cologne, Germany) for the NIR-monitored runs and using a Christ model Epsilon 2-4 lyophilizer (Salm en Kipp, Breukelen, The Netherlands) to prepare the samples for stability testing and FTIR measurements. Initially the shelf was precooled at 3 °C. Subsequently the samples were frozen by lowering the shelf temperature to -40 °C at 0.5°/min. The samples were kept at this temperature for 150 minutes whilst the pressure was lowered to 150 μBar after which the shelf temperature was increased to -20 °C at 0.5°/min. This temperature was maintained for 23 hours, completing primary drying. Secondary drying was initiated by raising the temperature to 40 °C in 350 minutes, maintaining the pressure of 150 μBar. After 6 hours, or more for some of the NIR-probed lyophilization cycles (Fig. 2), the cycle was ended. The samples for storage and FTIR were prepared using a similar cycle and the vials of those runs were stoppered at 150 μBar inside the freeze-drier. It should be noted that here the terms primary and secondary drying are used to describe the phase of the freeze-drying process as is common in literature. The terms primary and secondary drying usually reflect removal of free ice and more tightly adsorbed water, respectively. Therefore, a clear distinction will be made between primary and secondary drying conditions, which refer to the process parameters during freeze-drying, and sublimation of free ice (primary drying) and removal of tightly bound water (secondary drying), referring to the drying of the product.

2.4 Near-Infrared Spectroscopy

2.4.1 NIR Measurement

The in-line Near-Infrared measurements were conducted as described previously (Pieters et al., 2012). Briefly, a fiber-optic non-invasive probe and a Fourier transform NIR spectrometer (Thermo Fisher Scientific, Nicolet Antaris II near-IR analyzer) were used to collect diffuse

reflectance NIR spectra of the sample every minute during lyophilization. Spectra were collected between 10 000 and 4 500 cm^{-1} with a resolution of 8 cm^{-1} and were the average of 32 scans. The probe measured the vial from the side at the bottom, measuring over a detection area of around 28 mm^2. The probe thus did not measure the complete cake, but it was assumed the exposed part was representative for the entire sample.

2.4.2 NIR Data processing

Data were processed using in-house written m-files in Matlab 7.1 (The Mathworks, Natick, MA, United States of America) as previously reported (Pieters et al., 2012). In brief, spectra were preprocessed by offset correction, vector normalization and the second derivative with 17 point Savitzky-Golay smoothing. The frequency of the minimum of the second derivative of the amide A/II band ($V_{A/II}$) (near 4850 cm^{-1}) was monitored to evaluate changes in the hydrogen bonding strength of the protein's amide groups. A decrease in this frequency is indicative of a relative increase in hydrogen bonding, and vice versa (Katayama et al., 2009; Liu et al., 1994). Using spline interpolation, missing frequency values were predicted, achieving a data spacing of 1 cm^{-1} to allow better accuracy. The frequency values of 10 previous measurements were averaged to reduce noise. The intensity of the band near 5160 cm^{-1} in the baseline-corrected and normalized spectra was used to calculate an apparent water absorbance (AWA) value (Pieters et al., 2012; Rantanen et al., 2001). The band at that frequency is the product of OH-stretching and HOH bending vibrations and indicates unfrozen water moiety and provides an estimation of the apparent water loss. During the first hours of primary drying conditions, present ice interfered with the spectra. These spectra were therefore not taken into account and the obtained $V_{A/II}$ and AWA values thus represent the second part of primary drying conditions and secondary drying conditions. Effectively this means that the actual primary drying (e.g. removal of free ice) is not measured, instead secondary drying (e.g. removal of tightly absorbed water) is monitored under the conditions

184 commonly referred to as primary (e.g. low shelf temperature, low pressure) and secondary
185 freeze-drying (high shelf temperature, very low pressure). Logically, the water removed under
186 secondary freeze-drying conditions is the most tightly bound water.

187 **2.4.3 NIR Data Interpretation**

188 The change in AWA intensity and the frequency of the amide A/II band were plotted
189 against process time. This plot provides information protein-environment interactions after the
190 bulk of the free ice has been removed and how these change during the process. A low A/II
191 frequency at the start of the process, by definition at 0 change in AWA, indicates good initial
192 interactions between the protein and its environment (e.g. sugars, water). No conclusions can
193 be drawn from the final A/II frequency nor from the exact A/II values, because the AWA has
194 not been quantitatively related to remaining water activity and residual moisture after
195 lyophilization and it is not the same for the different formulations. Comparing the relative
196 changes in A/II and AWA curves during the different parts of drying, however, do provide
197 useful information.

198 The part of the curves up to 1656 minutes corresponds to primary drying conditions,
199 followed by a part corresponding to secondary drying conditions, in which the temperature is
200 increased relatively fast, ending with an isothermal phase. If the hydrogen bonds taken up by
201 water are replaced effectively by sugar, the curve of the A/II frequency tracks the AWA
202 curve, meaning the A/II frequency rises when water is removed. Diverging AWA and A/II
203 curves on the other hand point at incomplete water replacement. Water substitution is most
204 important during removal of tightly bound water (secondary drying), because degradation of
205 LDH during lyophilization mainly occurs under secondary drying conditions (Luthra et al.,
206 2007). How the A/II and AWA curves track each other under secondary drying conditions is
207 therefore particularly interesting.

2.5 Fourier Transform Infrared Spectroscopy

In addition to the in-line NIR measurements, FTIR was used to monitor interactions between protein and sugar in the solid state and to investigate whether conformational changes occurred during storage. The samples were measured in solid form after lyophilization. Between lyophilization and analysis, samples were stored at -20°C in the stoppered vials to prevent any degradation. For these measurements, a Tensor 27 FTIR spectrometer (Bruker Optics, Ettlingen, Germany) was used, equipped with a liquid nitrogen cooled mercury cadmium telluride detector. The spectrometer was placed inside a glove bag (Sigma AtmosBag), which was purged with dry nitrogen gas to avoid moisture sorption of the samples. The vials were only opened inside the glove bag, where the relative humidity was below 5%. Spectra were collected between 4 000 and 850 cm^{-1} at a resolution of 4 cm^{-1}, and for every measurement 256 scans were obtained and averaged. Additionally, the equipment itself was purged using dried nitrogen gas to eliminate the influence of atmospheric gasses. Samples were measured at room temperature without further preparation using a high temperature golden gate mark II attenuated total reflectance accessory (Specac, Slough, United Kingdom). A few milligrams of sample were used for each measurement. Opus software (Bruker, Ettlingen, Germany) was used for processing of the data. Spectra were baseline corrected and triplicate measurements were averaged. The storage stability samples were vector normalized between 1720 and 1590 cm^{-1} (amide I region) and this region was plotted. Additionally, the second derivative of this band was calculated using 17 point Savitzky-Golay smoothing and plotted. Using the same second derivative, frequencies of peak and shoulders in the Amide I regions were determined for the various protein-sugar ratio samples. These frequencies (averaged of n=2, each measured in triplicate) were plotted against sugar content for all formulations. The absorption of the various sugars in the used amide I region was negligible.

2.6 Storage stability

To investigate the stabilizing effects of the different excipients, a storage stability test was performed. Samples were stored at 60 °C for up to 28 days in closed vials and LDH functionality was tested at various time points (after 1,3,7, 14 and 28 days) during that period, using an enzymatic activity assay. After storage at 60 °C, up until the moment of analysis, samples were stored at -20°C. It was assumed that during storage at -20°C, no significant degradation of LDH occurred. Samples were reconstituted with 2 mL of a solution of 0.01% bovine serum albumin in 0.1 M phosphate buffer (pH=7.5) and diluted 40 times twice to reach a concentration suitable for the assay. The assay is based on the rate of conversion of pyruvate into lactate by LDH by measuring the decrease in absorption at 340 nm caused by a decreasing amount of substrate NADH. The assay was carried out as previously described (Tonnis et al., 2015).

3. Results

3.1 Near-Infrared during lyophilization

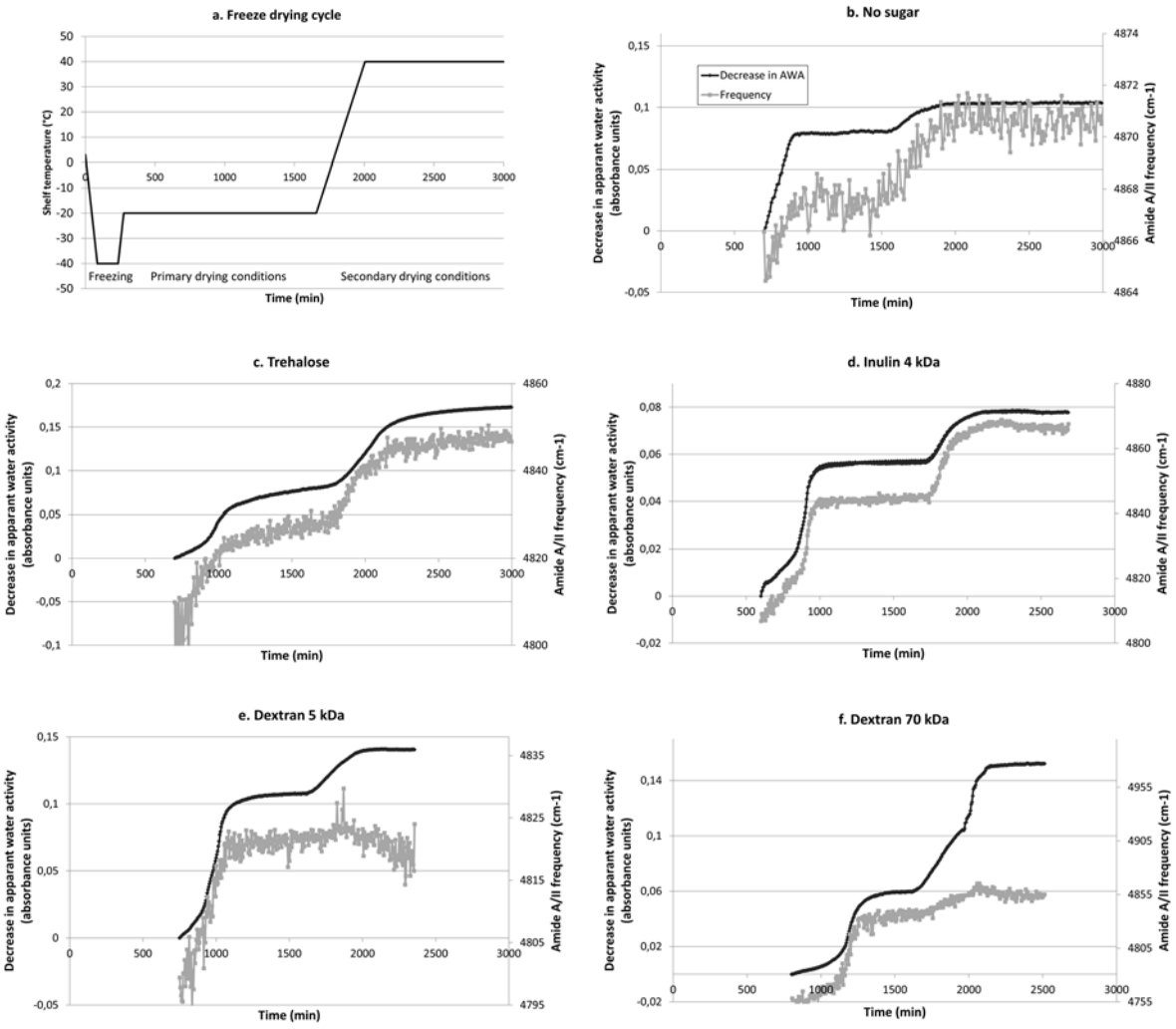


Fig. 2. An overview of the shelf temperature used during lyophilization (a) Amide A/II frequency and decrease of Apparent Water Absorption (AWA) of the different formulations during lyophilization (b-f).

The AWA and A/II frequencies were monitored during lyophilization for each formulation, the results are depicted in Fig. 2. The top right Figure shows the progress of the shelf temperature during the cycle and illustrates which part of lyophilization occurs at which time.

The AWA and A/II curves of the sample without sugar show similar changes throughout the process. The A/II frequency (tracking hydrogen bonds formed with the protein amide groups) increases with a further decrease of the AWA (tracking water loss), which indicates that the protein gets partially dehydrated as a result of the drying process. This effect is larger under

secondary drying conditions than under primary drying conditions, as more energy is provided for the removal of bound water when the temperature is raised. The total change in $V_{A/II}$ is only 6 cm^{-1} for the sample without sugar, four times smaller than that of the sugar containing formulations, showing less changes in hydrogen bonding during the process. The A/II frequency at 0 AWA (start of the monitoring) is also much higher than the other formulations, indicating that less intermolecular interactions between protein and surroundings are present at that moment. For the formulations containing trehalose and inulin the curves show very similar profiles, with the curves running parallel both under primary and secondary drying conditions. For both dextran formulations (of 5 and 70 kDa respectively), the AWA and A/II curves run parallel under primary drying conditions, but under secondary drying conditions the decrease in AWA goes up further, whilst the A/II frequency decreases several cm^{-1} instead of increasing. Compared amongst each other, the larger dextran shows a larger overall shift in A/II frequency.

3.2 FTIR of lyophilized samples

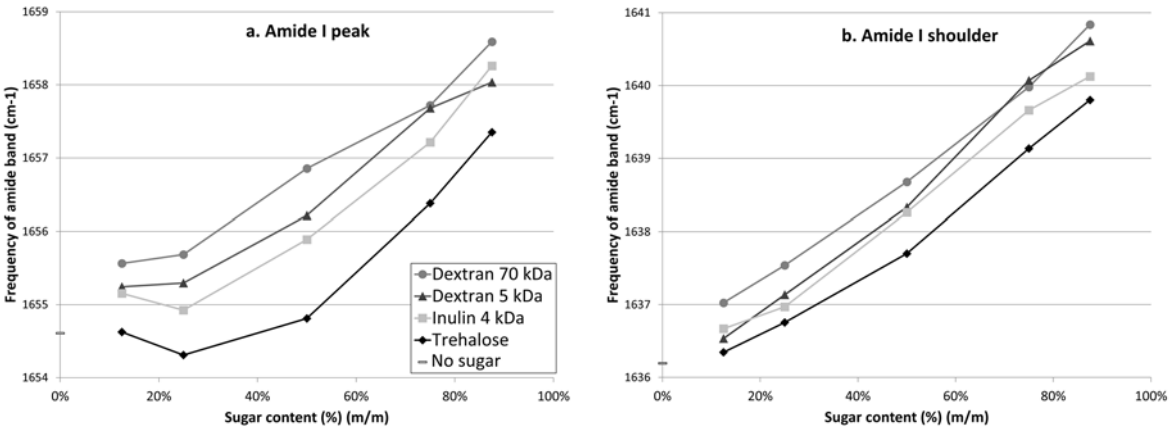


Fig 3. Frequency of amide I peak (a) and shoulder (b) of LDH of the different formulations at varying protein-sugar ratios.

The frequencies of the peak and shoulder in the amide I region of the formulations with varying protein-sugar ratio are plotted against sugar content in Fig 3. In most cases, the

frequency of both the peak and shoulder go up with increasing sugar content, indicative of a reduction of the density of hydrogen bonding of the protein in the formulation. In the shoulder area this seems a linear process, whereas for the amide I peak there is less increase or even a small decrease in frequency up to 25% sugar content. Trehalose has the lowest frequencies, indicative of strongest hydrogen bonding, followed by inulin, dextran 5 kDa and lastly dextran 70 kDa. The differences in frequencies between formulations are relatively insensitive to sugar content, showing similar spacing for different types of sugar at all contents. This is mostly true for the amide II shoulder.

3.3 Storage stability: Activity

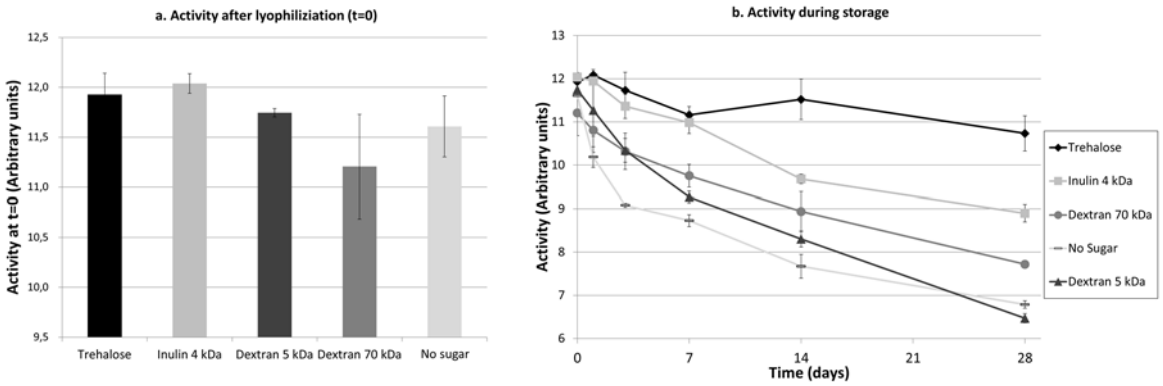
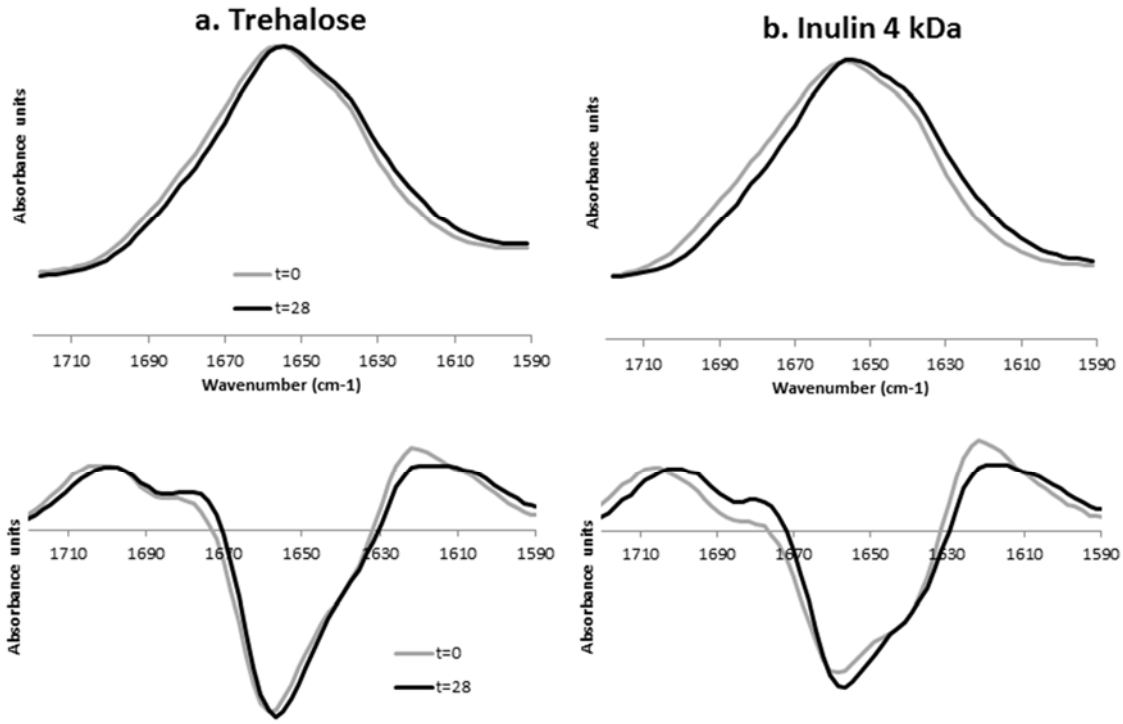


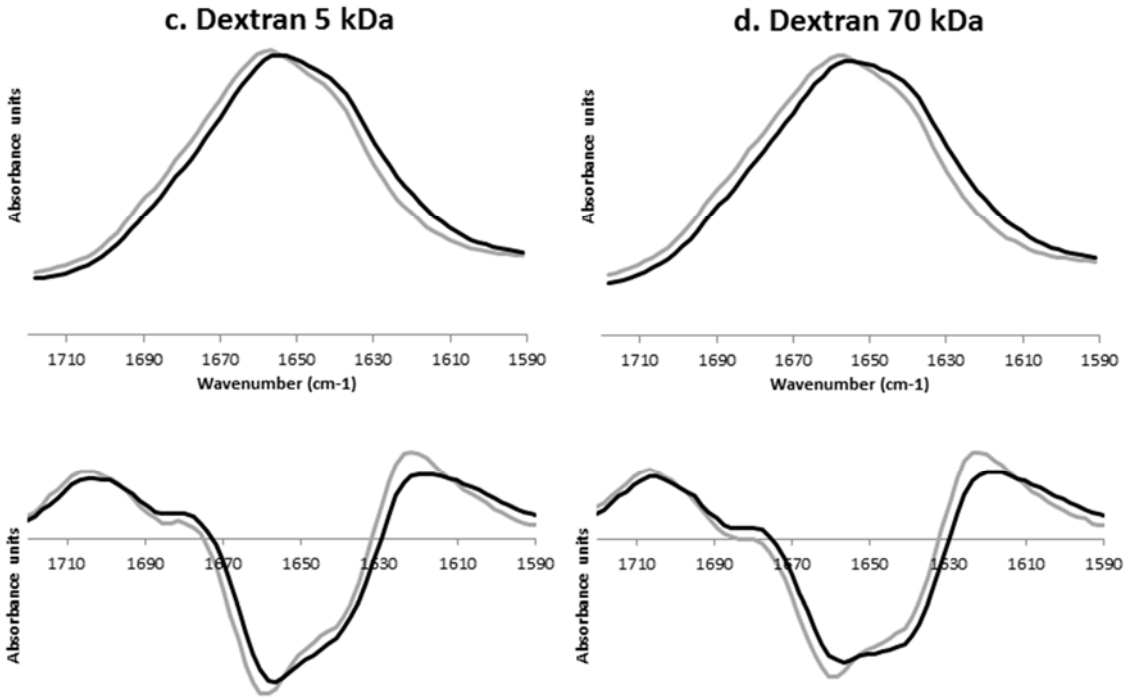
Fig 4. Activity of lactate dehydrogenase samples immediately after lyophilization (a) and during storage at 60 °C for 28 days (b)

The activity of LDH of the various formulations was monitored both immediately after lyophilization and after subsequent storage at 60 °C (Fig. 4). Immediately after lyophilization, the differences in activity of the different formulations were small. It should be noted that the left panel is a zoomed in portion of the right panel, exaggerating small differences. The activity of the dextran 70 kDa formulation was lower than the other formulations. However, this difference was not significant (t-test, $p = 0.12$) compared to the reference without sugar. To prevent skewing of the data, the activity during storage (Fig. 4b) is therefore represented

as measured (in arbitrary units) rather than relative to the activity at t=0. Trehalose was best capable of maintaining protein stability, with only a 10 ±3% overall loss in activity compared to t=0. Inulin 4 kDa was the second best stabilizer showing a loss of 26 ±2% after 28 days. The dextrans lost 47 ±1% (5 kDa) and 36 ±1% (70 kDa) activity after 28 days of storage. Dextran 5 kDa basically does not act as a stabilizer at all.

3.4 Storage stability: Secondary structure





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307 Fig 5. Normalized amide I band and second derivative of that band of the different
308 formulations before and after storage at 60°C for 28 days. Gray lines represent before storage,
309 black after storage.

310 Fig. 5 depicts the amide I bands of LDH before and after storage of the various
311 formulations. For all formulations, there is a peak around 1660 cm^{-1}, indicating alpha helix
312 structures, and a shoulder near 1640 cm^{-1}, showing beta-sheets (Jackson and Mantsch, 1995).
313 The shoulder increases in size during storage for all formulations. The peak at 1660 cm^{-1}
314 remains similar during storage for trehalose, increases slightly for inulin stabilized LDH and
315 decreased for dextran based formulations. The band also shows a small shift to lower
316 wavenumbers during storage, more so for both dextrans than for the other formulations.
317 Overall trehalose shows least spectral changes, followed by inulin 4 kDa, whereas dextran 5
318 kDa and dextran 70 kDa displayed most changes during storage.

319

4. Discussion

In this study, the influence of hydrogen bond formation between protein and sugars during lyophilization in relation to the storage stability of a model protein, LDH, was investigated. Previously, it was hypothesized that smaller and more molecular flexible sugars are better stabilizers of proteins during storage as they were less affected by steric hindrance in interacting with the protein, provided that they remained vitrified (Tonnis et al., 2015). Therefore, smaller and more molecularly flexible sugars are expected to be better capable of forming hydrogen bonds with the protein. The sugars used were a disaccharide (trehalose), two similarly sized oligosaccharides: molecularly flexible inulin 4kDa and rigid dextran 5 kDa, and a large molecularly rigid polysaccharide (dextran 70 kDa).

As described in the methods section, the NIR results provide information about the formation of hydrogen bonds between the protein and surroundings during the different stages of lyophilization, in particular during the removal of tightly bound water (secondary drying). By comparing the AWA and A/II throughout the process, one can see during which phase loss of hydrogen bonds is prevented, indicating lyoprotection. The relatively high starting A/II frequency of the formulation without sugar, compared to the sugar containing formulations, indicated either little intramolecular hydrogen bonding at the start or somestructural loss during the freezing and primary drying stage (prior to monitoring). Small structural changes should result in only little loss of activity, as was confirmed by activity tests directly after freeze-drying. It seems unlikely that the structural changes were completely reversible upon reconstitution, therefore it is most likely that the A/II frequency was high due to little hydrogen bonding between the protein and its environment. In addition to that, the change in Amide A/II frequency was small for the formulation without sugar compared to the sugar formulations, meaning there was only a small amount of hydrogen-bond formation during drying. As there is no stabilizer present, these few interactions are most likely hydrogen bonds

between the protein molecules. The changes in frequency occur simultaneously with the changes in AWA, thus these few bonds are formed throughout the entire lyophilization cycle. For the formulation with sugar, the initial amide A/II frequencies were lower, indicating that they were more able to compensate for the loss of protein-environment hydrogen bonds during earlier phases, i.e. freezing and primary drying.

The good correspondence between AWA and A/II signals and large overall shift in A/II frequency for formulations with trehalose and inulin are indicative of the formation of new hydrogen bonds between the protein and these sugars both under primary and secondary drying conditions. It should be noted that under primary and secondary drying conditions, only secondary drying was monitored as it was technically impossible to monitor the spectral changes during primary drying. Both dextrans are capable of replacing hydrogen bonds under primary drying conditions, but seem to fail to do so under secondary drying conditions, where the most tightly bound water is removed. The larger dextran has a larger shift in amide A/II frequency during removal of tightly absorbed water (secondary drying) compared to the smaller dextran, which could indicate a better interaction with the protein for the larger dextran.

The amide I band, mainly associated with C=O stretching of the amide group, is sensitive to both inter- and intramolecular hydrogen bonding (Barth, 2007). Generally a lower frequency is indicative of more or stronger hydrogen bonds (Jackson and Mantsch, 1995). Fig. 3 thus indicates that the small disaccharide trehalose has the best hydrogen bond forming potential with the protein, followed by oligosaccharides inulin 4 kDa, dextran 5 kDa and lastly polysaccharide dextran 70 kDa. This is true for each protein-sugar ratio tested. Remarkably, however, the amide I frequencies shift up with an increasing amount of sugar. A possible explanation for this is that the protein-sugar interactions are weaker than the protein-protein

interactions, resulting in weaker overall hydrogen bonding and therewith higher amide frequencies.

Storage stability results show similar trends compared to previously published results with 4 model proteins, despite the substantially higher protein-sugar ratio (1:10 versus 1:249) used here for the spectroscopic analyses (Tonnis et al., 2015). The results show that disaccharide trehalose conserves the activity of LDH best, followed by molecularly flexible oligosaccharide inulin 4 kDa. The two molecularly rigid dextran formulations perform worst, with dextran 5 kDa only slightly outperforming the formulation without sugar. Overall, dextran 5 kDa and the formulation without sugar lost nearly half of their activity during 4 weeks of storage, whereas the formulation with trehalose only lost around 10% activity. The FTIR spectra show lowest change during storage for trehalose, with more changes for inulin and most changes for both dextrans. Generally, spectral changes are undesired as the aim is to preserve the proteins native structure. Here too the smallest change to the amide I region correlate with the best conservation of activity. The changes in secondary structure, a decrease in alpha helix content and an increase in unordered structures or β-sheets, indicate aggregation (Jackson and Mantsch, 1995).

The sugars which maintained protein activity best, i.e. disaccharide trehalose and molecularly flexible inulin, distinguished themselves from the other tested sugars by their ability to replace hydrogen bonds throughout the lyophilization process. The more molecularly rigid dextrans, regardless of their size, were unable to efficiently replace hydrogen bonds under secondary drying conditions, leading to the formation of less or weaker hydrogen bonds than smaller or molecularly more flexible sugars. These findings are in line with the flexibility hypothesis (Fig. 1), showing that smaller and more molecularly flexible sugars are less affected by steric hindrance and can therefore interact with the protein better. The effect of size is larger than the effect of molecular flexibility. It should be noted that all

sugars used here had sufficiently high glass transition temperatures to maintain vitrification, which is also required for protein stabilization (Allison et al., 1999; Tonnis et al., 2015). The larger dextran 70 kDa is a better stabilizer than dextran 5 kDa. Dextran 70 kDa has a higher Tg than dextran 5 kDa meaning its ability to vitrify is bigger. However, as long as sufficient vitrification is achieved, a higher glass transition temperature does not further increase stability (Grasmeijer et al., 2013). A possible explanation for the difference between the differently sized dextrans could be the branching of dextran, which will have a greater influence at larger molecular weight. This could also be an explanation for the relatively large shift in amide A/II frequency for dextran 70 kDa. Alternatively, the Maillard reaction could be a reason for this difference, as the reducing end groups of dextran are relatively more present in lower molecular weight dextrans. Previous results, however, showed this same trend and ruled out the Maillard reaction as cause and additionally to that no browning was observed (Tonnis et al., 2015).

Interestingly, during lyophilization there is only very limited divergence in loss of activity, despite the clear differences in hydrogen bonding of the different excipients. It is during storage that these differences in stabilizing capacity become evident. As mentioned in the introduction, it is likely that the water replacement (protein-excipient hydrogen bond formation) translates to a reduced molecular mobility of the protein during storage, resulting in increased storage stability. In-line NIR measurements can therefore be a valuable addition in estimating storage stability of proteins, which is not apparent directly after lyophilization. Additionally, these results show that hydrogen bonding under secondary drying conditions is particularly important. Removal of the hydrogen bonds of this tightly bound water has a larger impact on the protein than the removal of less tightly bound water which is removed under primary drying conditions. A good stabilizer should thus be capable of forming hydrogen bonds both under primary as well as secondary drying conditions. Again it should be noted

that primary drying was not investigated due to interference of ice and these results thus describe hydrogen bond replacement during removal of tightly bound water (secondary drying) under primary and secondary drying conditions. This paper shows that in-line NIR during lyophilization is preeminently suitable to determine how the excipients behave during each phase of the lyophilization cycle and can provide useful insights in protein-excipient interactions.

5. Conclusion

This manuscript shows that, compared to larger and molecularly more rigid proteins, smaller and molecularly more flexible sugars are better able to form hydrogen bonds with a model protein during lyophilization. This is in support of the previously described flexibility hypothesis, which states that the sugars can overcome steric hindrance more effectively. The differences in hydrogen bonding mainly occur under secondary drying conditions, during which the most tightly bound water is removed. It should be noted that all sugars used here had sufficiently high glass transition temperatures to maintain vitrification, which is also required for protein stabilization. The differences in hydrogen bonding do not result in different activities after lyophilization for the different formulations, but they only become apparent during storage. During storage less hydrogen bonding results in a greater loss of activity. This is most likely the result of increased molecular mobility of the protein in the solid state, resulting in more aggregation. A good stabilizer should thus be capable of forming hydrogen bonds both under primary as well as secondary drying conditions. Lastly, in-line NIR can be a useful tool in gaining a deeper understanding of protein-excipient interactions during different phases of lyophilization.

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