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5 # ISOMALT AND ITS DIASTEREOMER MIXTURES AS STABILIZING
6 # EXCIPIENTS WITH FREEZE-DRIED LACTATE DEHYDROGENASE

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ABSTRACT

The purpose of this research was to study isomalt as a protein-stabilizing excipient with lactate dehydrogenase (LDH) during freeze-drying and subsequent storage and compare it to sucrose, a standard freeze-drying excipient. Four different diastereomer mixtures of isomalt were studied. The stability of the protein was studied with a spectrophotometric enzyme activity test and circular dichroism after freeze-drying and after 21 days of storage at 16% RH. Physical stability was analyzed with differential scanning calorimetry and Karl Fischer titration. Statistical analysis was utilized in result analysis. LDH activity was almost completely retained after freeze-drying with sucrose; whereas samples stabilized with isomalt diastereomer mixtures had a considerably lower protein activity. During storage the sucrose-containing samples lost most of their enzymatic activity, while the isomalt mixtures retained the protein activity better. In all cases changes to protein secondary structure were observed. Isomalt diastereomer mixtures have some potential as protein-stabilizing excipients during freeze-drying and subsequent storage. Isomalt stabilized LDH moderately during freeze-drying; however it performed better during storage. Future studies with other proteins are required to evaluate more generally whether isomalt would be a suitable excipient for pharmaceutical freeze-dried protein formulations.

Keywords: isomalt, freeze-drying, protein stabilization, lactate dehydrogenase, sucrose

1. INTRODUCTION

The number of proteins as active pharmaceutical ingredients in drug preparations has increased during the last decade and it will continue to grow in the future. Biological products accounted for 23% of worldwide prescription and OTC sales in 2014, with the corresponding number only 14% in 2006. The proportion is forecast to grow further, reaching 27% of all medicine sales worldwide in 2020 (EvaluatePharma 2015). Most of these biological products are monoclonal antibodies and recombinant products.

Because proteins are complicated and fragile molecules when it comes to their production, storage, and administration, developing protein pharmaceuticals is a challenge (Cromwell, et al. 2006). Many proteins are prone to chemical and/or physical degradation, which makes their pharmaceutical formulation development difficult (Wang, et al. 2007). If stability of the protein in an aqueous liquid formulation is a problem, often the best choice is a freeze-dried formulation. The stability of proteins in freeze-dried (lyophilized) formulations is better than in aqueous solutions because the degradation reactions can be generally slowed down to such an extent that the protein drug will stay stable for months or years (Chang and Pikal 2009). The structure of many proteins may change during freeze-drying, at least partially, if they are not stabilized with excipients such as disaccharides or sugar alcohols i.e. polyols (Ohtake, et al. 2011), which are able to form an amorphous phase during freeze-drying. This protects proteins from freezing- and drying-induced degradation reactions. In addition, disaccharides and polyols may stabilize proteins after freeze-drying in the dry state during storage. The excipients that are most commonly used for this purpose in protein formulations are sucrose and trehalose (Mensink, et al. 2017; Schwegman, et al. 2005).

Investigating new excipients that could be used as protective agents in freeze-dried pharmaceutical protein formulations is highly important because currently there are only a limited number of

64 excipients that can be used for this purpose. The excipients currently used are not always able to
65 stabilize all proteins sufficiently. Also, all stabilizing excipients have their own downsides since
66 even trehalose, which is considered as the golden standard in protein stabilization and a good
67 amorphous stabilizer, has been observed to crystallize during storage under frozen conditions
68 (Singh, et al. 2011; Sundaramurthi and Suryanarayanan 2010). Because the number of protein drugs
69 is going to increase in the future, broadening the range of excipients used for their formulation
70 development is important.

71 Isomalt is a well-tolerated, non-toxic polyol, which is traditionally used as a sweetening agent in the
72 food industry and as a tabletting excipient for pharmaceutical purposes (Ndindayino, et al. 2002;
73 Sáska, et al. 2010; Sentko and Willibald-Ettle 2012). Isomalt is included in the British (BP), the
74 European (PhEur) and the United States (USP-NF) pharmacopoeias as well as in Japanese
75 Pharmaceutical Excipients (JPE) (British Pharmacopoeia Commission 2015; European
76 Pharmacopoeia Online 9th Edition 2017; United States Pharmacopeial Convention 2014). Isomalt is
77 a mixture of two stereoisomers, 6-O-α-D-glucopyranosyl-D-sorbitol (1,6-GPS) and 1-O-α-D-
78 glucopyranosyl-D-mannitoldihydrate (1,1-GPM) (Sentko and Willibald-Ettle 2012). The structures
79 of the diastereomers are presented in Figure 1. The glass transition temperatures of these
80 stereoisomers are 66°C for 1,1-GPM and 55°C for 1,6-GPS (Cammenga and Zielasko 1996).
81 Isomalt does not belong to the group of reducing sugars that may cause an alteration in the degree
82 of glycation of proteins in freeze-dried formulations during storage (Leblanc, et al. 2016).

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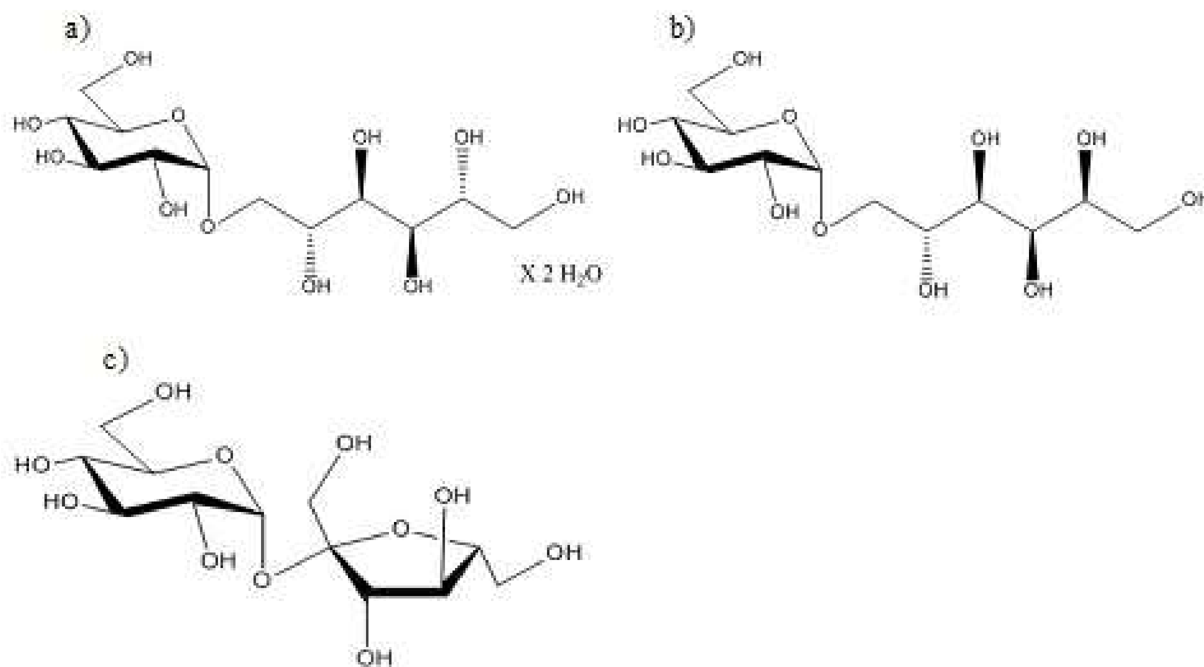

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85 **Fig. 1.** The structures of a) GPM and b) GPS (the two diastereoisomers of isomalt, together
86 comprising the polyol excipient) and c) sucrose
87

88 Acute toxicity of isomalt has been studied in rats and the LD_{50} dose was over 2500 mg/kg b.w. with
89 the intravenous and intraperitoneal administration routes (Musch, et al. 1973). The chronic toxicity
90 of orally administered isomalt as a part of diet has been studied in animals and it was found to be a
91 safe substance. It did not affect the mortality rate of the animals, and there was no evidence of
92 carcinogenicity, or adverse effects on fertility, reproductive performance or development (World
93 Health Organization 1987). Furthermore, no maternal toxicity occurred and effects on embryonic,
94 fetal development or reproductive performance were not observed (Smits-Van Prooije, et al. 1990;
95 Waalkens-Berendsen, et al. 1990a; Waalkens-Berendsen, et al. 1990b; World Health Organization
96 1987). Isomalt has also been administered orally to humans. No significant changes in serum levels
97 of glucose, insulin, lactic acid, hemoglobin, cholesterol, triglycerides, or high-density lipids were
98 observed (Thiebaud, et al. 1984; World Health Organization 1987). Hepatic and renal function tests
99 were within normal limits and no neurological and cardiovascular changes were observed after oral
100 administration (World Health Organization 1987).

101 To the best of our knowledge, no studies involving the use of isomalt as a protein stabilizing
102 excipient in freeze-dried formulations have been published. The physical properties of isomalt and
103 the fact that it is not a reducing sugar make it an interesting excipient to be studied in this context.
104 Some polyols, such as mannitol and sorbitol, tend to crystallise during freeze-drying and/or storage
105 (Al-Hussein and Gieseler 2012; Costantino, et al. 1998; Kadoya, et al. 2010), which reduces their
106 ability to stabilize proteins. Previously, we have characterized the solid state changes of isomalt and
107 its diastereomer mixtures during freeze-drying and storage in the absence of proteins (Koskinen, et
108 al. 2016). It was found that crystalline isomalt could be transformed into the amorphous form with
109 freeze-drying, and that during storage at up to 16% RH amorphous isomalt had good physical
110 stability. The mixtures containing both diastereomers of isomalt, GPM and GPS, remained
111 physically more stable than a mixture containing only the GPM diastereomer. The amorphous
112 nature and good physical stability, which are important qualities for protein-stabilizing sugar
113 excipients, further increase the interest of studying whether isomalt is able to stabilize proteins
114 during freeze-drying and storage.

115 The aim of this study was to investigate the ability of isomalt, in four different diastereomer ratios,
116 to stabilize proteins during freeze-drying and subsequent storage with the model protein lactate
117 dehydrogenase (LDH). The cryo- and lyostabilizing effects of isomalt mixtures are evaluated by
118 protein activity measurements and secondary structure analysis. LDH is a widely used model
119 protein in freeze-drying related protein studies, and its activity can be quantified with a simple
120 spectrophotometric *in vitro*-test (Chatterjee, et al. 2005; Cochran and Nail 2009). Sucrose, a
121 traditionally used cryo- and lyoprotecting excipient in freeze-dried protein formulations, was used
122 as a reference in this study. The chemical structure of sucrose is presented in Figure 1. In addition to
123 the fact that sucrose is a relevant reference material because of its wide use in lyophilized protein
124 pharmaceuticals, it also shares some similar physicochemical properties with isomalt, such as
125 molecular mass, glass transition temperature, solubility in water at higher temperatures (over 50°C),

126 heat of fusion, heat of solution, and viscosity and water activity of aqueous solutions (Sentko and
127 Willibald-Ettle 2012).
128

129 **2. MATERIALS AND METHODS**
130

131 **2.1 Materials**
132

133 L-Lactic dehydrogenase from rabbit muscle in ammonium sulfate suspension (product number
134 L2500), sucrose (product number 16104), and tris(hydroxymethyl)aminomethane (TRIS) (product
135 number T6066) were acquired from Sigma-Aldrich Co. (St. Louis, Missouri, USA). One molar HCl
136 (product number 30024.290) was obtained from VWR (Radnor, Pennsylvania, USA). Four different
137 grades of isomalt (galenIQ™ 721, galenIQ™ 720, Isomalt GM and pure GPM-stereoisomer) were
138 kindly donated by Beneo-Palatinit GmbH (Germany). By using the four supplied isomalt grades,
139 the GPM-content of the freeze-dried samples was varied from 23.5 to 99% with the GPS-content
140 varying from 0.0 to 75.7%. Thus approximate GPM:GPS isomer ratios of 1:3 (galenIQ™ 721), 1:1
141 (galenIQ™ 720), 2:1(Isomalt GM), and 1:0 (pure GPM-stereoisomer) could be studied. Highly
142 purified water (Milli-Q, Millipore Inc., USA) was used in all of the studies.

143 The chemical formula of sucrose is $C_{12}H_{22}O_{11}$ and those for isomalt are $C_{12}H_{24}O_{11}$ and
144 $C_{12}H_{24}O_{11} \cdot 2H_2O$ for the anhydrous and dihydrate forms, respectively. The molecular structures of
145 sucrose and isomalt diastereomers share a common group, a glucose monomer and they have a
146 similar molecular mass (Figure 1). Besides a glucose ring, sucrose has a fructose ring in its
147 structure, whereas GPM and GPS isomers contain mannitol and sorbitol monomers instead of
148 fructose, respectively.

149

150 **2.2 Preparation of the LDH solutions, freeze-drying and stability studies**

151

152 The acquired LDH suspension was initially dialyzed in 0.5 M TRIS-HCl buffer solution (pH 7.4) to
153 remove the ammonium sulfate prior to its use. This involved diluting 8 ml of the LDH ammonium
154 sulfate suspension with 8 ml of TRIS-HCl buffer and transferring the suspension to a Spectra/Por ®
155 Biotech regenerated cellulose dialysis membrane, with molecular weight cut off 25 000 Da
156 (Spectrum Laboratories Inc, Rancho Dominguez, California, USA). The membrane had previously
157 been pretreated by soaking in purified water for 25 minutes. The resulting protein solution was
158 dialyzed for 24 h in TRIS-HCl buffer and the buffer was replaced three times during the dialysis.

159 The dialyzed protein solution was diluted with 0.5 M TRIS-HCl buffer to produce a solution with
160 an LDH concentration of 0.5 mg/ml. The concentration of the dialyzed protein stock solution was
161 determined spectrophotometrically with UV-1600PC Spectrophotometer (VWR, Radnor,
162 Pennsylvania, USA) at 280 nm. The different isomalt grades or sucrose were added to the protein
163 solution to produce a 100 mg/ml concentration of excipients, thus corresponding a 1:200
164 protein/excipient ratio. Protein solution with no added sugar excipient was also produced. Each of
165 the produced solutions (1 ml) was pipetted into 20 ml freeze-drying vials and freeze-dried using a
166 Lyostar II freeze-dryer (SP Industries Inc., Warminster, USA). The vials were first equilibrated at
167 20°C for 10 minutes and then at 0°C for 30 minutes. The samples were then frozen at -40°C for 2
168 hours. The freezing ramp rate was 1°C/min. Primary drying was performed with a shelf temperature
169 of -30°C and pressure of 150 mTorr for 18 hours. Secondary drying was carried out by heating the
170 chamber 0.1°C/min to 35ºC, while retaining the pressure at 150 mTorr. Maintaining the pressure in
171 the same level during primary and secondary drying was found to work with isomalt samples in a
172 previous study (Koskinen, et al. 2016). After the freeze-drying cycle was complete, the chamber

173 temperature was lowered from 35ºC to 25°C and the chamber was filled with N_2 gas to bring the
174 pressure inside the chamber back to atmospheric pressure. The vials were then stoppered in the N_2
175 atmosphere inside the chamber by compression before opening the chamber door.

176 The vials intended for storage stability studies were transferred into a desiccator containing a
177 saturated salt solution of lithium chloride, thus producing a relative humidity of approximately
178 16%. In our previous study even this low relative humidity condition was observed to cause stress
179 to the physical stability of freeze-dried isomalt samples stored in the desiccator without stoppers
180 (Koskinen, et al. 2016), and was thus chosen to this current study. The vials were opened inside the
181 desiccator and were stored without stoppers at ambient temperature of approximately 22°C. The
182 humidity percentage inside the desiccator was monitored with a Tinytag data logger (Gemini Data
183 Loggers, Chicester, United Kingdom). The samples were stored in the desiccator for 21 days.

184

185 **2.3 Protein activity measurements**

186

187 LDH activity was measured using a lactate dehydrogenase activity assay kit (product number
188 MAK066) acquired from Sigma-Aldrich Co. (St. Louis, Missouri, USA). The freeze-dried samples
189 were first rehydrated with 1 ml of purified water and then diluted to an LDH concentration of 0,005
190 mg/ml and pipetted into a Nunc MicroWell 96-well plate (Thermo Fisher Scientific, Massachusetts,
191 USA), after which the reagents of the activity assay kit were added to the wells. The measurements
192 were performed at 37ºC with a Varioskan Flash spectrophotometer (Thermo Fisher Scientific,
193 Massachusetts, USA) and the readings were collected with SkanIt software (Thermo Fisher
194 Scientific, Massachusetts, USA). Each sample was studied in triplicate from different vials (n=3)
195 and two parallel wells were made from each vial. Thus the protein activity results are presented as
196 mean values of six measurements. The mean well-to-well variation between duplicate samples was

197 14,21% (minimum 0,09%, maximum 81,41%). LDH activity was measured before freeze-drying,
198 after freeze-drying and after 3 weeks of storage. LDH activity of the lyophilized samples was
199 compared to the activity of unprocessed LDH by calculating the relative activity remaining after
200 processing.

201

202 **2.4 Differential scanning calorimetry**

203

204 Differential scanning calorimetry (DSC) was used to monitor the thermal behavior and solid state
205 properties of the lyophilized samples and raw materials. The freeze-dried LDH samples were
206 studied after freeze-drying and after 3 weeks of storage. Approximately 3-7 mg of the sample was
207 transferred in an aluminum DSC pan and closed with a pierced lid to allow free water evaporation
208 during measurements. Each sample was made in triplicate and the samples were taken from
209 different vials (n=3). The DSC experiments were carried out by first equilibrating the samples at
210 25ºC for 3 minutes and then heating them at 10ºC/min to 180ºC for isomalt samples, or to 160ºC for
211 sucrose samples, in order to avoid sample spillage from the pans. The runs were carried out under
212 50 ml/min N_2 purge. The results were analyzed with STAR^e Thermal Analysis Software (Mettler-
213 Toledo International Inc., Greifensee, Switzerland).

214

215 **2.5 Circular dichroism**

216

217 Circular dichroism (CD) was used to study if the secondary structure of LDH changes during
218 freeze-drying or storage. The freeze-dried samples were rehydrated with 1 ml of purified water and
219 diluted to one fifth of the original concentration. The CD spectra of the samples were measured

with a Jasco J-720 spectrometer (Jasco Inc., Easton, Maryland, USA). Samples were scanned in the far UV range from 200 to 250 nm at a speed of 50 nm/min, an increment of 1 nm and a response time of 1 s. A quartz cuvette with a path length of 1 mm was used and each final spectrum was an averaged from three consecutive scans. The spectra were smoothed with a Savitzky-Golay function using a convolution width of 13 points. The measured average ellipticity was converted to mean residue ellipticity (MRE) ($[\theta]_{mrw,\lambda}$ [deg cm^2 $dmol^{-1}$]) using the equation (Kelly, et al. 2005)

$$[\theta]_{mrw,\lambda} = (MRW \cdot \theta_\lambda)/(10 \cdot d \cdot c)$$

where MRW was mean residue weight, using 111.2 g/mol for the studied protein, θ_λ was the measured average ellipticity, d was pathlength (cm), and c was concentration (g/ml). The CD spectrum of LDH was measured before freeze-drying, after freeze-drying, and after 3 weeks of storage. Also the spectrum of denatured LDH was measured after equilibrating it for 10 min with 1 M HCl. In addition to that, the excipient solutions without LDH were studied in order to verify that the excipients did not interfere with the CD spectrum of LDH in the studied UV region.

2.6 Water content measurements

The residual water content of the freeze-dried LDH samples was measured with Karl-Fischer titration (KF). The measurements were carried out after freeze-drying and during storage with a V30 Volumetric KF Titrator (Mettler-Toledo International Inc., Greifensee, Switzerland). The KF measurements were conducted from the same sample vials as the DSC experiments. Rubber stoppers were immediately placed on the vials when taking them out of the desiccator and all possible steps were taken to minimize ambient moisture uptake between DSC and KF analyses, specifically the vials were stoppered immediately after taking out powder samples from the vials and the analyses were conducted as rapidly as possible. The lyophilization vial was weighed, the

residual sample amount was poured into the KF titration vessel and the vial was weighed yet again to calculate the sample mass. The water content results are presented as an average of three sample vials.

2.7 Statistical analysis

Results obtained from the LDH activity measurements were analyzed using independent samples Kruskal-Wallis test. The analyses were performed on the results of different sample vials (n=3) of each treatment (unprocessed, freeze-dried without sugars or freeze-dried with isomalt/sucrose). Analyses were performed using IBM SPSS Statistics software version 23.0 (IBM Corp., Armonk, NY, USA). For all analyses, p-values of <0.05 were considered to be statistically significant.

3. RESULTS AND DISCUSSION

3.1 Secondary structure

CD spectroscopy was performed on the unprocessed protein as well as on the freeze-dried protein formulations with excipients before and after storage. The measured CD spectrum of unprocessed LDH with its two ellipticity minima at around 209 and 222 nm agree well with previous CD studies of LDH (Kouassi, et al. 2007; Mi, et al. 2002; Mi and Wood 2004). Based on the CD spectroscopy measurements, the secondary structure of LDH freeze-dried with sugar excipients was altered to some extent (Figure 2 a, b). However, an absence of isomalt or sucrose precipitated more significant

266 LDH secondary structure changes, displayed as a higher increase in MRE. A reference CD
267 spectrum of non-lyophilized LDH denatured with HCl shows a complete destruction of the
268 secondary structure of LDH. Overall, both isomalt and sucrose had a significant effect in preserving
269 the secondary structure of LDH during lyophilization. All LDH samples with different sugar
270 excipients had a very similar CD spectrum. As such, no clear differences could be resolved in the
271 secondary structure of LDH stabilized with either different isomalt grades or sucrose (Figure 2 a).

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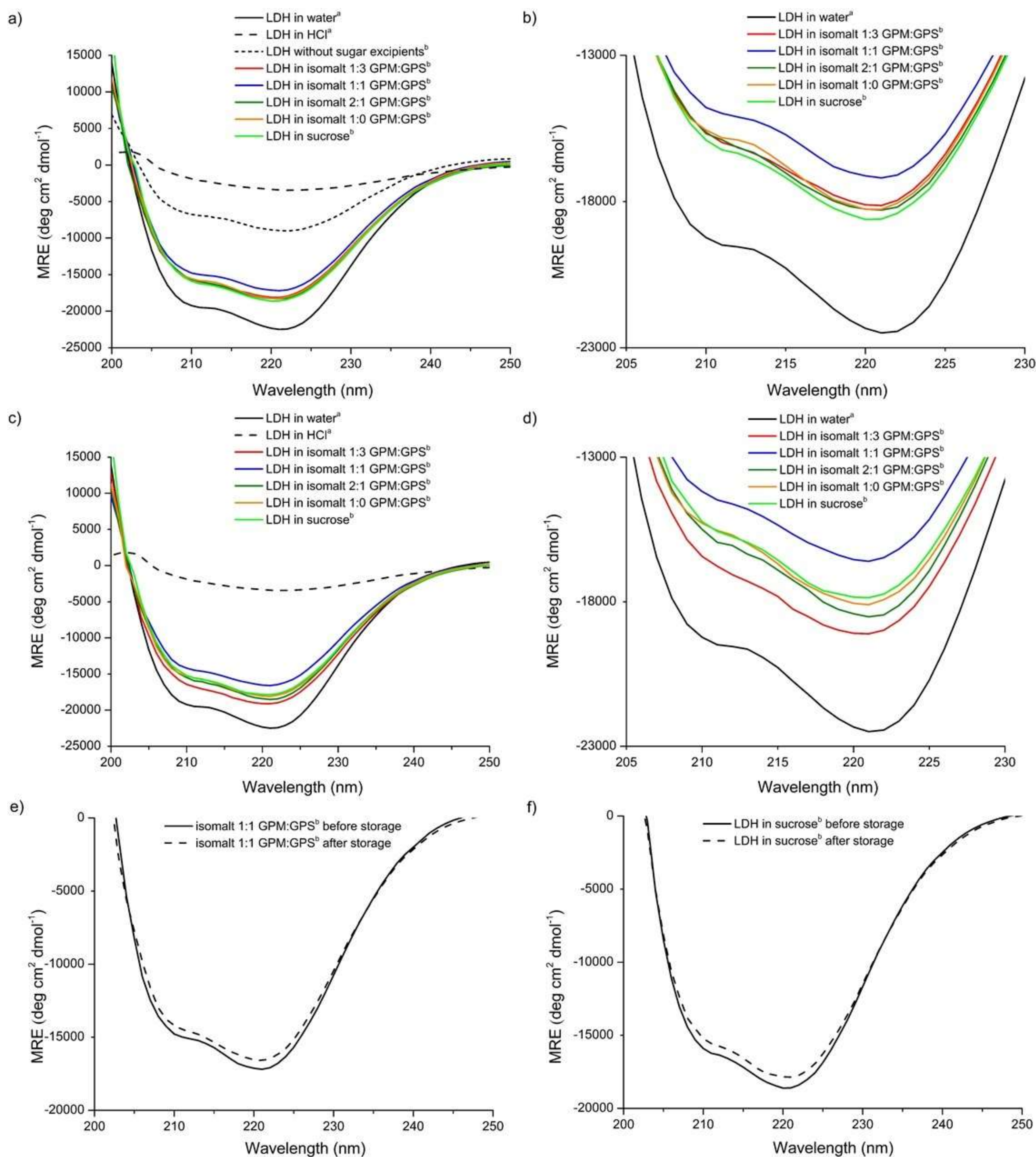

273
274 **Fig. 2.** The CD spectra of a) LDH samples after freeze-drying with zoom in (b), c) LDH samples
275 after 3 weeks of storage at 16% RH with zoom in (d), LDH before and after storage with e)1:1
276 GPM:GPS and f) sucrose. Reference spectra of unprocessed LDH in water and denatured LDH in
277 HCl are included. [a] non-lyophilized samples [b] lyophilized samples
278
279

280 The CD spectra of isomalt- and sucrose-containing samples after 3 weeks of storage were slightly
281 altered, suggesting that some changes to the LDH secondary structure occurred during storage

282 (Figure 2 c-f). The two ellipticity minima around 209 and 222 were not as distinct as in the spectra
283 of both untreated LDH and lyophilized samples with sugars before storage. As an example of the
284 samples having the most changes during storage, the CD spectra of LDH with 1:1 GPM:GPS and
285 sucrose before and after storage are illustrated in Figures 2 e and f. The predominant secondary
286 structure of LDH is α-helix with 39.2% of the residues in this conformation, 22.4% in β-sheet
287 conformation and 38.4% in other secondary structures (Kouassi, et al. 2007). Loss in ellipticity
288 observed with LDH both after freeze-drying and after storage with and without the sugars resulted
289 in decrease in the α-helix content and increase in the content of β-sheet and other conformations
290 (Kouassi, et al. 2007). When comparing the spectra of after-storage samples containing different
291 isomalt grades or sucrose to one another and to the spectrum of unprocessed LDH, only small
292 differences could be observed between the excipients. After storage, the isomalt grade containing a
293 ratio of 1:3 GPM:GPS had the smallest increase in MRE compared to the spectrum of unprocessed
294 LDH, and the spectrum of 1:1 GPM:GPS had the highest increase in MRE. The spectra of two other
295 isomalt grades, 2:1 and 1:0 GPM:GPS, and sucrose were in between the former two in regards to
296 their increase in MRE. Based on this, the isomalt grade with a 1:1 GPM:GPS ratio did not preserve
297 the secondary structure of LDH during storage as well as the other isomalt grades or sucrose.
298 Because the differences between isomalt and sucrose were overall quite small, it can be concluded
299 that during freeze-drying and subsequent storage at 16% RH isomalt and sucrose preserved the
300 secondary structure of LDH equally well.

301

302 **3.2 Protein activity**

303

304 The measured LDH activity of samples lyophilized with sugars varied depending on the excipient.
305 Samples containing sucrose as the stabilizing excipient had the highest LDH activity after freeze-

306 drying (Figure 3). The four different isomalt grades showed slight variation between the GPM:GPS
307 diastereomer mixtures, the 1:3 GPM:GPS samples having the highest LDH activity after
308 lyophilization. During storage, the LDH activity decreased in all samples with sucrose-containing
309 samples having the highest absolute loss in LDH activity.

310 The enzymatic activities of different samples were analyzed with Kruskal-Wallis test to observe
311 statistically relevant differences between the groups. A difference was observed between groups of
312 different treatments before the storage period ($p < 0.0004$). Based on the results of LDH activity
313 results and statistical analysis, sucrose stabilized LDH best and samples stabilized with isomalt or
314 containing no sugar excipients showed lower protein activities compared unprocessed LDH and
315 sucrose-containing samples.

316 During storage LDH activity decreased in all samples. Sucrose-containing samples had the highest
317 absolute loss in LDH activity during storage, from 95.6 to 37.2% (relative to unprocessed LDH
318 activity). Between different isomalt grades the variation in LDH activity loss was relatively small,
319 though 2:1 GPM:GPS-containing samples retained their LDH activity the best, the loss being only
320 1.5% units, from 52.1% before storage to 50.6% after storage. Compared to the samples containing
321 no stabilizing excipients, all sugar-containing samples retained a higher level of LDH activity after
322 storage. When no sugars were included in the sample, LDH activity was lost almost completely
323 with only 10.9% remaining after storage (Figure 3). As in the case of statistical analysis of pre-
324 storage results, Kruskal-Wallis test observed a difference between the different groups after the
325 storage period ($p = 0.006$). Unprocessed LDH was not included in the analysis, so only post-storage
326 activity results of freeze-dried samples were compared to each other. This suggests that some or all
327 sugar excipients stabilized LDH better compared to samples containing no isomalt/sucrose.

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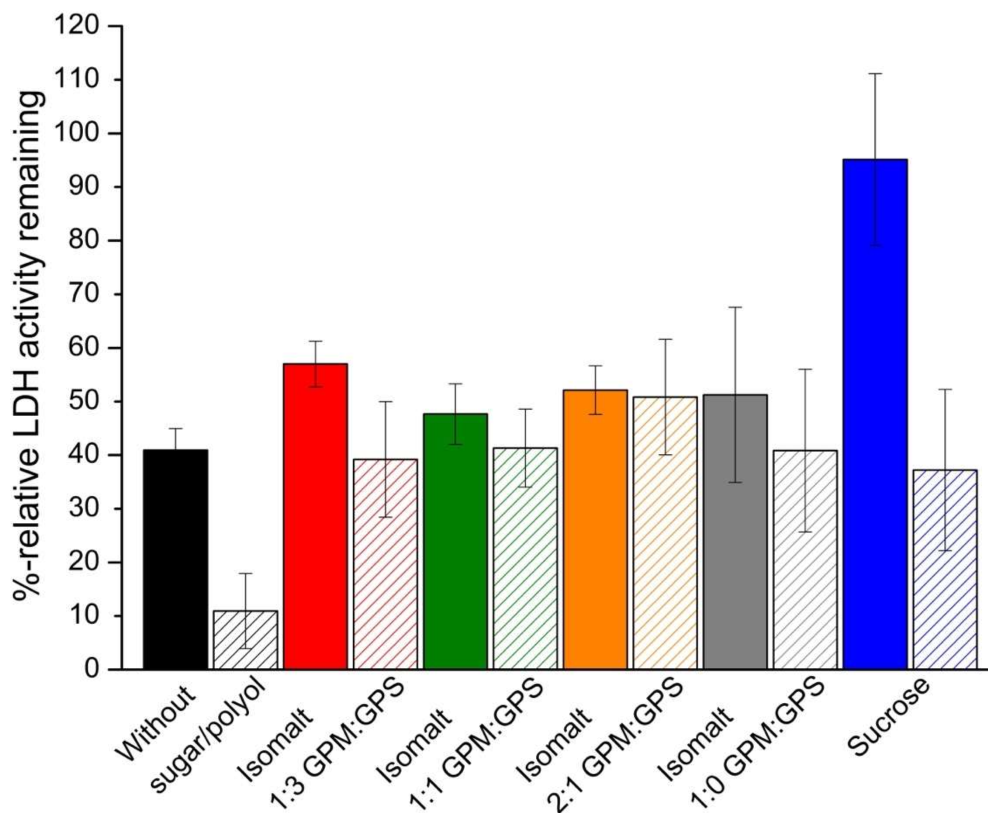

329
330 **Fig. 3.** Relative LDH activity after freeze-drying (solid bars) and after storage (dashed bars) with
331 and without sugar excipients (mean ± sd)
332

333 In previous studies, when LDH has been freeze-dried without any stabilizing excipients, LDH
334 activity recoveries between 22.2 and 68.5% have been recorded, and the remaining LDH activity
335 after lyophilization of 40.9% observed in this current study falls in the middle range of these
336 previous observations (Anchordoquy, et al. 2001; Kadoya, et al. 2010; Luthra, et al. 2007). The
337 concentration of LDH itself also has an effect on its recovery after lyophilization, for it has been
338 observed that as the concentration increases, the remaining LDH activity also increases
339 progressively (Anchordoquy and Carpenter 1996). In a previous study, the recovery of LDH
340 increased approximately from 20 to 55% as the LDH concentration was varied between 25 and 500
341 µg/ml, respectively (Anchordoquy and Carpenter 1996). Primary drying does not appear to be a
342 critical step in the freeze-drying process of LDH, for no activity loss was observed even in the
343 absence of any stabilizing excipients, thus secondary drying seems to be the critical phase in the

344 lyophilization process with respect to retaining LDH activity (Jiang and Nail 1998; Luthra, et al.
345 2007).

346 The difference between sucrose and the four isomalt grades in their stabilizing effects during
347 lyophilization are better resolved from the LDH activity measurements than from the CD
348 spectroscopy measurements. The ability of sucrose to preserve a higher activity level of LDH
349 during freeze-drying was not observed in the CD spectra when comparing sucrose to isomalt, for
350 their spectra were very similar compared to each other. LDH freeze-dried without sugar excipients
351 had the lowest remaining activity after freeze-drying and this confirms the CD spectroscopy results,
352 which indicated greater alterations in the secondary structure of LDH lyophilized without sugars.

353 The observed loss in LDH activity during storage in samples containing sugars is confirmed by the
354 CD spectroscopy results as the spectra measured after storage lost some of their shape in the two
355 ellipticity minima compared to pre-storage spectra. The better ability of isomalt to stabilize LDH
356 during storage compared to sucrose observed from the results of protein activity measurements did
357 not show as clear differences in their CD spectra measured after storage. Based on the measured CD
358 spectra, the loss in activity during storage was partly due to alterations on the secondary structure of
359 LDH, but also other factors presumably had an effect on the loss of protein activity, especially in
360 the case of LDH stabilized with sucrose, which lost most of its activity during storage. Aggregation
361 is one of the most important reasons to cause physical instability to proteins and it may be one
362 possible reason of causing loss in activity during storage in this present investigation (Wang, et al.
363 2007).

364 Out of all the studied excipients, sucrose was the best cryo- and lyostabilizing excipient. All isomalt
365 grades stabilized LDH during freeze-drying producing higher LDH activity values compared to
366 LDH freeze-dried without any sugar excipients, 1:3 GPM:GPS stabilizing LDH best out of the
367 different isomalt grades. Especially during storage, the protein stabilizing effects of different

368 isomalt grades were observed. The protein samples containing isomalt preserved LDH activity
369 better during storage compared to the samples without any sugar excipients. Sucrose has been used
370 as a cryo- and lyostabilizing excipient with LDH in previous studies, and as in this current study,
371 sucrose has been able to successfully retain a high LDH activity after freeze-drying and it is also
372 observed to stabilize LDH after freeze-thawing (Anchordoquy, et al. 2001; Kadoya, et al. 2010;
373 Luthra, et al. 2007). Sucrose in concentrations of 0.025 mol/l, 0.1 mol/l and 0.5 mol/l with an LDH
374 concentration of 0.25 mg/ml, thus corresponding approximate protein/excipient ratios of 1:34, 1:137
375 and 1:685, retained 60.4%, 87.1% and 84.7% LDH activity after freeze-drying, respectively
376 (Anchordoquy, et al. 2001). In another study, 50 mg/ml sucrose resulted in almost complete LDH
377 activity recovery after freeze-drying (94.0%), but lower concentrations (0.05, 0.5 and 5 mg/ml)
378 resulted in no stabilization or only partial activity recovery (Luthra, et al. 2007). Unfortunately, the
379 ratio of protein to excipient cannot be calculated, as the LDH concentration of the samples was not
380 specified. In the current study, all of the studied sugars stabilized the protein against destabilizing
381 effects of moisture during storage better than when no sugars were included in the samples. Isomalt
382 performed better than sucrose during storage, for the loss in LDH activity in samples containing
383 different isomalt grades was only moderate compared to sucrose-containing samples. The protein
384 activity of samples stabilized with sucrose decreased considerably during storage as a result of the
385 effect of moisture. In a previous study, at high storage temperatures (40 or 60ºC) sucrose with a
386 protein/excipient ratio of 1:1 000 was not able to stabilize LDH when the moisture content of the
387 samples was high (5.6% w/w), but at a lower storage temperature of 20ºC it performed well as a
388 stabilizing excipient, retaining LDH activity in the same level before and after 90 days of storage
389 (Kawai and Suzuki 2007). Compared to these previous results, the inferior ability of sucrose to
390 stabilize LDH during storage in this current study can result from the five times lower
391 protein/excipient ratio used in this study. A very high protein/excipient ratio of sucrose (1: 2 000)

392 has been found to stabilize LDH at a high storage temperature of 50ºC during a 7-day storage
393 period (Kadoya, et al. 2010).

394 The mechanisms by which disaccharides and polyols stabilize proteins during the stresses caused by
395 lyophilization have been widely studied, although the discovered mechanisms are not universally
396 approved and are still under more in-depth investigation. Multiple different mechanisms have been
397 found to affect protein stabilization. In liquid state and during the freezing stage of lyophilization,
398 sugars and polyols stabilize proteins by the preferential exclusion mechanism, which means that the
399 stabilizing excipients are preferentially excluded from the surface of the protein as the protein
400 molecules prefer to interact with water molecules (Timasheff 1998). This thermodynamically
401 stabilizes multimeric proteins, like LDH, against stress-induced dissociation, because LDH can
402 undergo low-temperature-induced subunit dissociation that leads to irreversible structural changes
403 (Anchordoquy, et al. 2001; Jaenicke 1990). A high concentration of sucrose or isomalt used in this
404 current study increases the viscosity of the solutions, which can also stabilize LDH during the
405 freezing stage by restricting diffusion of the solute molecules and minimizing the rate of chemical
406 reactions (Hagen, et al. 1995). During freeze-drying, sucrose and isomalt are transformed from
407 crystalline excipients to amorphous (Carstensen and Van Scoik 1990; Koskinen, et al. 2016), which
408 enables them to stabilize LDH during the drying stage of lyophilization by forming a glassy matrix,
409 in which the protein is stabilized by reduction of conformational changes and by conformational
410 relaxation (Hagen, et al. 1995). The formation of a viscous glassy matrix is considered to be one of
411 the most important stabilization mechanisms of sugars and polyols in general (Hagen, et al. 1995;
412 Kadoya, et al. 2010). Despite this, according to one previous study, it appears that formation of a
413 glassy matrix is not important for protection of LDH during freezing (Anchordoquy, et al. 2001).
414 According to another previous study, preferential exclusion during freezing and vitrification during
415 drying combined with water substitution might explain how sucrose is able to stabilize LDH during
416 freeze-drying (Luthra, et al. 2007). Another important stabilization mechanism of sugars is

hydrogen bonding (Allison, et al. 1999). Sucrose, along with other sugar excipients, is able to hydrogen-bond to dried proteins, and because of this they are able to inhibit the loss of LDH activity during dehydration by preventing protein unfolding (Anchordoquy, et al. 2001). Isomalt and sucrose share one common group, a glucose monomer, in their structures (Figure 1). Besides a glucose ring, sucrose has a fructose ring in its structure, whereas GPM and GPS isomers contain mannitol and sorbitol monomers instead of fructose. The differences in hydrogen bond formation between isomalt or sucrose and a protein therefore result from the latter part of the structures. Both isomalt and sucrose contain OH-groups that can hydrogen bond to proteins. Theoretically, sucrose and isomalt could both stabilize LDH by hydrogen bond formation equally well, but since protein stabilization mechanisms are still under speculation, hydrogen bond formation is only one of many mechanism behind protein stabilization. According to Allison et al. (1999) hydrogen bonding efficiency between proteins and sugars varies according to their structures, and differences between sugars in their ability to stabilize proteins may be partially due to differences in the extent and intimacy of hydrogen bond formation. Despite similarities in the molecular structures of sucrose and isomalt, it cannot be excluded that differences in the extent and intimacy of hydrogen bond formation to LDH could exist. This may be one explanation why sucrose stabilized LDH better than isomalt during freeze-drying.

3.3 Physical properties

The freeze-dried samples did not have a perfect cake structure. In particular, the samples containing sucrose and isomalt in 1:3 or 1:1 GPM:GPS ratios had collapsed, suggesting that the freeze-drying cycle could have been further optimized. The protein activity after freeze-drying increased in the order; without sugar/polyol < 1:1 GPM:GPS < 1:0 GPM:GPS < 2:1 GPM:GPS < 1:3 GPM:GPS <

441 sucrose. Therefore despite their collapse during lyophilization, samples containing sucrose and 1:3
442 GPM:GPS had the highest protein activities. In previous studies with collapsed lyophilizates it was
443 observed that collapse during freeze-drying did not negatively affect stability, and even better
444 protein stability was observed in collapsed cakes compared to non-collapsed (Fonte, et al. 2014;
445 Schersch, et al. 2010). After storage, the LDH activity had decreased the most relative to pre-
446 storage activity in the order; without sugar/polyol > sucrose > 1:3 GPM:GPS > 1:0 GPM:GPS > 1:1
447 GPM:GPS > 2:1 GPM:GPS. Based on this, it may be possible that the observed collapse during
448 freeze-drying in samples containing sucrose, 1:3 GPM:GPS and 1:1 GPM:GPS may have had a
449 negative effect on their storage stability, although in a series of studies with several proteins,
450 including LDH, it was observed that lyophilizate collapse during freeze-drying did not negatively
451 affect protein stability even during long-term storage; however collapse during storage was
452 associated with decreased protein stability (Schersch, et al. 2010; Schersch, et al. 2013; Schersch, et
453 al. 2012). No additional collapse was observed in this current study during storage.

454 The freeze-dried samples were studied with DSC before and after storage to study their solid state
455 properties, including confirming whether they were amorphous or crystalline. All samples measured
456 after freeze-drying and 3 weeks of storage exhibited a glass transition temperature (T_g). This and the
457 absence of a crystallization exotherm and a melting endotherm suggest that they were amorphous
458 after freeze-drying and remained so during the 3 weeks of storage at 16% RH (Table 1). The
459 samples containing sucrose had the highest T_g (63.5ºC) after freeze-drying. After storage the T_g was
460 approximately 10ºC lower (53.7ºC) due to the moisture content rising from 1.9% before storage to
461 3.0% after storage. A T_g of 68.5°C has previously been measured for pure, dry sucrose (Urbani, et
462 al. 1997).

463 The T_g of samples containing isomalt varied depending on the ratio of GMP and GPS. After freeze-
464 drying, the samples containing a 1:3 ratio of GPM and GPS had the lowest T_g of 40.3ºC, and the T_gs
465 of other isomalt grades were 49.3, 51.5 and 58.9ºC for samples containing 1:1, 2:1 and 1:0

466 GPM:GPS ratios, respectively. As might be expected, and also observed in a previous study with
467 amorphous isomalt (Koskinen, et al. 2016), the samples' residual moisture contents had an effect on
468 their T_gs: as the T_g of isomalt samples grew higher, their moisture contents decreased. After freeze-
469 drying, residual moisture contents of 3.6, 3.1, 2.6 and 2.2% were recorded for the 1:3, 1:1, 2:1 and
470 1:0 GPM:GPS ratios, respectively. The T_gs of pure isomalt grades have been studied previously,
471 with values of 42.4, 51.4, 54.4 and 62.4ºC being reported for the 1:3, 1:1, 2:1 and 1:0 ratios of
472 GPM:GPS, respectively (Koskinen, et al. 2016).

473 During storage at 16% RH, the water contents of the samples increased by 0.4–1.1 percentage units
474 (Table 1). Consequently, the T_gs after storage were lower than pre-storage values, at 36.7, 40.5,
475 45.1 and 48.7ºC for the 1:3, 1:1, 2:1 and 1:0 GPM:GPS ratios, respectively. In a previous study,
476 freeze-dried pure isomalt samples containing the GPM:GPS ratios of 1:3, 1:1, 2:1 and 1:0 and
477 stored at 16% RH for 23 days exhibited T_g values of 39.3, 41.5, 43.3 and 45.3ºC, respectively
478 (Koskinen, et al. 2016).

479 Compared to the pure isomalt samples in similar storage conditions, which exhibited crystallization
480 peaks in the thermograms of 1:3 and 1:0 GPM:GPS samples (Koskinen, et al. 2016), no other
481 thermal events, except dehydration, were detected after storage in the measured thermograms of this
482 current study. The isomalt T_g and moisture content changes during storage did not predict the
483 changes observed in LDH stability during storage. Having said this, the 2:1 GPM:GPS mixture,
484 which stabilized the protein best, exhibited a modest decrease in T_g and rise in residual water
485 content when compared to sucrose samples, the T_g of which decreased markedly with a concurrent
486 large increase in water content, suggesting that the protein stability could partly be due to changes
487 in physical stability.

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494 **Table 1**. Glass transitions and residual water contents (mean ± sd, n =3)

	T_g (midpoint)	Residual water content
	[ºC]	(w/w)
1:3 GPM:GPS		
After freeze-drying	40.3±2.2	3.6%
After storage	36.7±1.8	4.0%
1:1 GPM:GPS		
After freeze-drying	49.3±1.3	3.1%
After storage	40.5±3.9	3.8%
2:1 GPM:GPS		
After freeze-drying	51.5±4.3	2.6%
After storage	45.1±2.7	3.2%
1:0 GPM:GPS		
After freeze-drying	58.9±1.9	2.2%
After storage	48.7±3.1	2.8%
Sucrose		
After freeze-drying	63.5±2.4	1.9%
After storage	53.7±2.9	3.0%

495

496 Isomalt diastereomer mixtures and sucrose are crystalline before lyophilization (Carstensen and
497 Van Scoik 1990; Koskinen, et al. 2016). The crystallinity of the isomalt/sucrose and TRIS mixtures
498 before freeze-drying was confirmed by performing DSC on the mixtures of the different
499 unprocessed isomalt grades and sucrose mixed with TRIS (Table 2). Sucrose and TRIS exhibited
500 only one peak at 135.7ºC (below the maximum measured temperature of 160ºC), which was
501 appointed as melting of TRIS since we measured also pure TRIS, which exhibited a melting peak at
502 138.8°C (onset 134.4°C). A melting point of 191.5ºC has previously been measured for D-sucrose,
503 although it also starts to decompose around this temperature (Hurtta, et al. 2004). Out of the four

504 different isomalt mixtures, 1:3, 1:1, and 1:0 GPM:GPS showed three endothermic peaks, while 2:1
505 GPM:GPS showed two peaks (Table 2). All four isomalt and TRIS mixtures had a melting peak of
506 TRIS at approximately 135ºC. Previously, for pure isomalt diastereomer mixtures endotherms
507 around 100ºC were interpreted as dehydration (observed with 1:3, 1:1 and 2:1 GPM:GPS),
508 endotherms at 128ºC-135ºC as dissolution of anhydrous GPM and GPS (observed with 1:1 and 2:1
509 GPM:GPS), an endotherm at 151ºC as melting of anhydrous GPM and GPS (observed with 1:3
510 GPM:GPS), an endotherm at 112ºC as melting of GPM dihydrate (observed with 1:0 GPM:GPS),
511 and an endotherm at 127ºC as dissolution/melting of GPM anhydrate (observed with 1:0
512 GPM:GPS) (Koskinen, et al. 2016). In the present study, the endotherms recorded below 100ºC for
513 all isomalt and TRIS mixtures are likely due to dehydration based on their shape and temperature
514 range. The other peaks at 129.8ºC (1:3 GPM:GPS), 128.2ºC (1:1 GPM:GPS), and at 109.7ºC (1:0
515 GPM:GPS) can be due to similar events as observed in the thermograms of their pure diastereomer
516 counterparts listed above (Koskinen, et al. 2016). Having said this, appointing the cause of the
517 endotherms is not completely undisputable, given that the samples are mixtures of two excipients,
518 which can have an interaction.

519

520 **Table 2**. DSC thermal events observed for unprocessed isomalt/sucrose and TRIS mixtures

	Endotherm 1		**Endotherm 2**		**Endotherm 3**	
	Onset [ºC]	Peak [ºC]	Onset [ºC]	Peak [ºC]	Onset [ºC]	Peak [ºC]
1:3 GPM:GPS	69.7	90.7	121.1	129.8	133.3	135.7
1:1 GPM:GPS	70.6	87.1	118.2	128.2	133.8	135.8
2:1 GPM:GPS	83.8	96.6	133.2	135.4	-	-
1:0 GPM:GPS	72.3	103.8	104.7	109.7	132.9	135.5
Sucrose	133.0	135.7	-	-	-	-

521

522 The different isomalt samples remained physically stable during the storage period, which is likely
523 to be one of the reasons why isomalt mixtures were able to stabilize the protein activity during
524 storage. Also, in previous studies, amorphous isomalt has exhibited high physicochemical stability

525 at different humidities and temperatures, which supports its potential as a freeze-drying excipient in
526 protein formulations (Koskinen, et al. 2016; Lipiäinen, et al. 2016).

527 The inferior ability of sucrose to preserve enzymatic activity of LDH during storage may be due to
528 a number of reasons. The alterations in secondary structure of LDH, as revealed by CD, explain
529 partly why sucrose was unsuccessful in preserving LDH activity during storage. The T_g of sucrose-
530 containing samples decreased from 63.5 to 53.7ºC, which can also reflect decrease in LDH stability,
531 as reduced protein stability may be linked with the decrease of T_g closer to storage temperature.
532 This is because a lower product T_g enables greater protein molecular mobility and thus increases
533 protein reactivity (Chang, et al. 2005a; Pikal, et al. 1991). High hydrogen bond formation between
534 freeze-dried amorphous sucrose and LDH has been observed to result in efficient protein
535 stabilization during storage, which deteriorated when the crystalline nature of sucrose increased
536 (Suzuki, et al. 1999). In this current study however, the DSC measurements did not indicate
537 crystallization of sucrose (no crystallization peak was observed), which would have been a clear
538 reason for inefficient stabilization during storage. Since amorphous sucrose is hygroscopic
539 (Carstensen and Van Scoik 1990), the moisture content of the samples was increased during storage
540 and this may also have an inferior effect on the protein stability. Due to an increase in the water
541 content, the viscosity of the amorphous phase of sucrose samples might have been decreased and
542 consequently protein degradation rate could have increased. On the other hand, similar moisture
543 contents have been found to be optimal for protein stability in a sucrose-containing IgG1 antibody
544 formulation (Chang, et al. 2005b), so the effect of moisture uptake is not straightforward. In a
545 previous study, it has been observed that the residual moisture of the protein sample has an effect on
546 the activity of freeze-dried LDH. High residual moisture was associated with high relative activity
547 after freeze-drying, with a residual moisture of 20% or higher yielded almost 100% relative activity
548 of lyophilized LDH (Jiang and Nail 1998). In the study however, the protein was freeze-dried in
549 buffer solution without any other excipients. Moisture contents as high as these can cause other

550 stability problems in a freeze-dried protein formulation containing stabilizing excipients during
551 storage because in the solid state water can act as a reactant and also dramatically lower the T_g of
552 the formulation (Chang, et al. 2005b). In this current study, residual water contents of only 1.4–
553 3.6% were measured after freeze-drying, depending on the sugar excipient included in the
554 formulation. In literature, in a saccharide-containing protein formulation water uptake during
555 storage increased molecular mobility in the glassy matrix but did not clearly increase the
556 aggregation rate of the protein with water content increase (Chang, et al. 2005b). For a lyophilized
557 IgG1 antibody formulation a moisture content of 2-3%, which falls in the same region as the
558 samples in this current study exhibited after lyophilization, was optimal for protein stability and
559 provided a minimum protein aggregation rate out of the water contents studied (0-5%) (Chang, et
560 al. 2005b).

561

562 **4. CONCLUSIONS**

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564 Both sucrose and isomalt were able to stabilize LDH during freeze-drying to some extent as
565 evidenced by the lower relative enzymatic activity in samples containing no sugar excipients.
566 Sucrose performed clearly better than isomalt as a cryo- and lyostabilizing excipient preserving
567 LDH activity almost fully after freeze-drying. However, during storage the samples stabilized with
568 sucrose lost over half of their initial activity, whereas isomalt protected LDH better from the
569 storage-induced destabilizing effects. The low protein activity level after storage suggests that
570 sucrose was not able to stabilize LDH efficiently in the presence of moisture. During storage, the
571 significance of stabilizing sugar excipients was emphasized as LDH samples without sugars lost
572 their activity almost completely, with only a quarter of the pre-storage LDH activity remaining after
573 the storage period. Even though isomalt was not the most optimal stabilizing excipient for the

574 studied protein during lyophilization, it showed some protein-stabilizing effects, especially during
575 the storage stability studies. In future studies, the aggregation tendency of lyophilized LDH
576 stabilized with isomalt should be investigated to assess whether aggregation takes place and does it
577 affect LDH stability. In order to assess the potential of isomalt as a novel cryo- and lyoprotecting
578 excipient in freeze-dried protein formulations, more freeze-drying studies should be carried out with
579 different proteins and in combinations with other excipients. Before isomalt could be used as an
580 excipient in freeze-dried formulations intended for parenteral use, also its intravenous toxicity must
581 be studied.

582

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593

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595

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597

598 **7. REFERENCES**

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